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An analysis of accelerated atherosclerosis in the hyperglycemic hyperlipidemic Syrian F(1)B hamster

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AN ANALYSIS OF ACCELERATED ATHEROSCLEROSIS IN THE HYPERGLYCEMIC HYPERLIPIDEMIC SYRIAN F_1B HAMSTER

BY

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THESIS

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ABSTRACT

AN ANAYLSIS OF ACCELERATED ATHEROSCLEROSIS IN THE HYPERGLYCEMIC HYPERLIPIDEMIC SYRIAN F_1B HAMSTER

By

Adele Jeanne Marone

University of New Hampshire, May, 2007

Humans with diabetes mellitus are two to five times more likely to develop cardiovascular disease and do so at a much younger age than non-diabetics. This study utilized the Syrian F_1B hamster, a model of human atherosclerosis, to: 1. determine whether one intraperitoneal injection of streptozotocin (STZ) could induce a diabetic-like hyperglycemic state, 2. compare the development of atherosclerotic lesions between non-treated (N), hyperglycemic (G), hyperlipidemic (L) and combined hyperlipidemic/hyperglycemic (L+G) hamsters, and 3. determine whether lesion development was accelerated due to plasma levels of glucose, triglycerides, total cholesterol, non-HDL-C, HDL-C, or lipid hydroperoxides, or by the TC/HDL-C or TG/HDL-C ratio or the expression of MDA or LOX-1 in the vascular wall of the hamster aortic arch. Plasma, and aortic arch and pancreatic tissue were collected and analyzed at 10 and 20 weeks.

Hamsters receiving one intraperitoneal injection of 40 mg/kg body weight of STZ had significantly elevated glucose levels compared to non-STZ injected hamsters. They also exhibited another characteristic of human diabetes, the atherogenic lipid triad characterized by hypertriglyceridemia, increased small dense LDL, and by week 20

decreased HDL-C levels. The lack of dependence on insulin, and its apparent continued secretion from the beta cells in the STZ treated hamsters appears to be reflective of human type II diabetes with the L+G hamster exhibiting the conditions of both insulin resistance and insulin impairment. The L+G hamsters also exhibited extremely accelerated atherosclerosis while the G only hamster exhibited no lesion development. Hyperglycemia and lipidemia alone independently induced MDA and LOX-1 expression. The combination of hyperglycemia/hyperlipidemia could potentially increase the expression of MDA and LOX-1 requiring further investigation to quantitate these levels. Atherogenesis appeared to be mainly associated with increased TC and non-HDL-C. While hyperglycemia alone does not appear to induce atherogenesis it may contribute to the acceleration of atherosclerosis when combined with elevated numbers of small dense LDL particles, with oxidation a major contributing factor to the formation of such particles. Our findings suggest that the L+G Syrian F_1B hamster is a useful model for determining the mechanism(s) involved in the development of accelerated atherosclerosis under the conditions of hyperglycemia.

INTRODUCTION

Cardiovascular disease (CVD) has been the primary cause of death in the United States (US) every year except one since 1900. CVD claims more lives in the US each year than the next five leading causes of death combined. In 2003 the next five leading causes of death were cancer, chronic lower respiratory diseases, accidents, diabetes mellitus, and influenza/pneumonia [1, 2]. Nearly 2,600 Americans die of CVD each day which averages approximately 1 death every 34 seconds and in 2001 CVD accounted for 38.5% of all deaths in the US.

According to a Center for Disease Control and National Center for Health statistics report, if all forms of CVD were eliminated, life expectancy would increase by almost 7 years. If all forms of cancer were eliminated, life expectancy would increase by only 3 years. According to the same study, the probability at birth of eventually dying from CVD is 47%. The statistics for other causes of death are: cancer – 22%, accidents – 3%, diabetes – 2 % and human immunodeficiency virus (HIV) - 0.7 % [1, 2]. These statistics demonstrate just how dominant CVD is as a cause of death in the US.

Based on 2003 population data, the average life expectancy of people born in the United States is now 77.5 years [2]. Recent declines in US death rates from CVD, due to new pharmaceuticals such as the statin drugs, and new surgical techniques, are largely responsible for the recent improvement in life expectancy. In the US, except for a relatively small increase in 1993, mortality from heart disease has steadily

declined since 1980 [1]. Even so, CVD is predicted to be the main cause of death not only in developed countries but worldwide by the year 2020. There are two major reasons for this. The first is the rapidly increasing prevalence of CVD in developing countries where there has been a decline in the communicable diseases that were previously the predominant cause of death. These countries are also beginning to adopt Western habits such as high fat diets and more sedentary lifestyles, both of which are major risk factors for CVD. The second and more underlying cause is the increasing prevalence of two other risk factors, obesity and its associated type II diabetes, particularly in the United States [3-12].

Atherosclerosis is the primary cause of CVD resulting in many deaths from heart attack and stroke. Many Americans already have atherosclerotic lesion formation by their early teens [13] as indicated by the presence of early atherosclerotic lesions in the coronary arteries of 50% of Americans between 10 and 14 years of age [14]. Atherosclerosis accounts for nearly three-fourths of all deaths from CVD. Therefore it can be stated that atherosclerosis alone is the leading cause of age-related morbidity and mortality in the Western world [15].

CHAPTER I

ATHEROSCLEROTIC LESION DEVELOPMENT

Atherosclerosis is a highly characteristic pathological response in specific arteries and at specific sites within those arteries. In humans, lesions occur most often in the abdominal aorta but also occur in the coronary and carotid arteries, in the arch and descending thoracic aorta, and in arteries in the legs. Lesions form in arteries that are generally larger than 2 mm, except in diabetics where lesions often may also form in smaller peripheral arteries. Areas of turbulent flow or low shear stress that tend to occur at arterial branch points are more prone to a thickening of the intima with age. Intimal thickenings in these areas may contain smooth muscle cells of both the contractile and the synthetic phenotype. Atherosclerosis is most likely to develop at these intimal thickenings and they are therefore called lesion prone areas. While lesion development is a continuous process of varying degrees it is generally characterized as having three distinct stages: early, intermediate and advanced.

Early Stage - Fatty Streak Lesions

The early stage lesion is called the fatty streak and as its name implies it is characterized by the deposition lipid in the arterial intima. These lipids are mainly cholesterol esters derived from circulating lipoproteins, particularly low density lipoprotein (LDL). Some factors that most likely contribute to this lipid

accumulation include (1) an increased plasma concentration of lipoproteins, especially LDL (2) endothelial dysfunction resulting in an increase in permeability allowing more of the smaller lipoproteins such as small, dense LDL particles to enter into the subendothelial space (3) aggregation of these particles due to interactions with extracellular matrix proteins and (4) entrapment of these particles due to a change in their physical characteristics such as an increased net negative charge.

In addition to intra- and extra-cellular lipid accumulation, monocyte infiltration is also a major characteristic of early stage lesion development. The adhesion of leukocytes to the endothelium in areas of increased permeability has been identified as one of the earliest events in atherogenesis [16]. There are three major groups of cellular adhesion molecules: the selectins, the immunoglobulin superfamily, and the integrins. All three of these adhesion molecule groups are involved in the multi-step process of monocyte migration and extravasation across the endothelium [17, 18]. These adhesion molecules are present on both endothelial cells (ECs) and leukocytes and each has a very specific role in one of the steps in this process.

The first step is the margination and rolling of monocytes in response to some insult or injury to the ECs. Selectins on both monocytes and ECs interact to form a weak, reversible bond causing the monocytes to slow and roll over the endothelium [19, 20]. Selectin expression may be induced by the cytokines interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) and bacterial endotoxin.

The next step is the firm adhesion of monocytes to ECs. The immunoglobulin superfamily adhesion molecules expressed on ECs responsible for this interaction are intercellular adhesion molecule-1 (ICAM-1) and vascular cellular adhesion molecule-1 (VCAM-1). The monocytes spread and then firmly adhere via binding of integrins on their surface, including very late antigen-4 (VLA-4) and lymphocyte function-associated antigen-1 (LFA-1) to VCAM-1 and ICAM-1 respectively on the ECs. ICAM-1 is constitutively expressed on ECs, but its expression can be increased by exposure to IL-1, interferon gamma (INF-γ), TNF-α, and bacterial endotoxin. VCAM-1 is upregulated by IL-1, TNF, bacterial endotoxin, and interleukin 4 (IL-4). While VCAM-1 expression on ECs is lower than ICAM-1 expression, VCAM-1 is the adhesion molecule most implicated in the pathogenesis of human atherosclerosis [21]. Data also suggests that the engagement of ICAM-1 and VCAM-1 induces intracellular signaling in the ECs which results in the opening of the endothelial cell-to-cell junctions. Engagement of ICAM-1 and VCAM-1 also induces reactive oxygen species (ROS) production in ECs [22, 23]. ROS may also interfere with endothelial integrity by oxidizing cysteine residues in tyrosine phosphatases which then impairs tyrosine phosphatase activity [24]. This also triggers a loss of endothelial cell-to-cell junctions resulting in an increase in EC permeability at these sites.

In the final steps of the process, the monocytes migrate through these endothelial junctions into the subendothelial space. In order for this movement to occur the monocytes have to form new adhesion sites and break old ones. Recently, junctional adhesion molecules (JAMs) present on both ECs and monocytes have

been shown to function as regulators of this transendothelial migration [25, 26]. platelet-endothelial cell adhesion molecule-1 (PECAM-1) is another junctional molecule present on both monocytes and ECs that may assist in this movement [27].

The transendothelial migration of monocytes is also driven by a chemotactic cytokine concentration gradient. Chemotactic cytokines, better known as chemokines, are a large family of small (8 to 14 kDa) extracellular ligands that interact with 7-transmembrane-spanning G-protein-coupled receptors [28] and are released by ECs, smooth muscle cells (SMCs) and monocytes already present in the artery. Monocyte chemoattractant protein-1 (MCP-1) is the chemokine most closely associated with atherogenesis. MCP-1 interacts via the C-C chemokine receptor-2 (CCR2) [29] on the surface of the monocytes.

The third major characteristic of early lesion development is foam cell formation. Once monocytes enter the subendothelial space they differentiate into macrophages. The principle function of macrophages is to engulf particles, internalize them and destroy them by phagocytosis or endocytosis. Macrophages internalize excess lipids present in the intima by phagocytosis. As they fill up with lipid droplets they take on a foamy appearance in histological sections. These lipid filled macrophages are referred to as foam cells [30].

Intermediate Stage - Fibro-muscular Lesions

Foam cells produce growth factors that stimulate SMCs in the media to proliferate and migrate into the subendothelial intima. The smooth muscle cells, in addition to engulfing lipid, also produce more proteoglycans and extracellular

matrix components. SMC proliferation and extracellular matrix (ECM) accumulation are characteristic of the intermediate or fibro-muscular stage of lesion development. All of the cells in the arterial wall are able to secrete cytokines that cause the SMCs in the media to migrate through openings in the internal elastic lamina and into the intima. These SMCs, along with the matrix molecules they secrete, form a cap overlying the accumulation of foam cells. It is at this point that atherosclerotic lesions are said to be in the intermediate or fibro-muscular stage.

Advanced Stage - Complicated Lesions

Eventually, lesions may progress to the advanced or complicated stage. These lesions are characterized by calcification, visible cholesterol crystals and a core of dead foam cells and gruel composed largely of cholesteryl esters. Stress occurs at the shoulder regions on either side of this necrotic core. It is at these weakened shoulder regions that rupture may occur releasing the highly thrombogenic gruel and tissue factor from the core. This leads to the formation of clots or thrombosis that may block the flow of blood within the artery causing the death of tissues downstream. When this occurs in a coronary artery the death of heart muscle results in a heart attack. When this occurs in the brain the death of brain tissue results in a stroke.

CHAPTER II

ATHEROSCLEROTIC RISK FACTORS

Risk factors related to the development of atherosclerosis were originally identified through epidemiological studies. The four main risk factors identified to date are high blood pressure, high total cholesterol, diabetes and smoking [31]. Other risk factors include increased age, physical inactivity, stress, a genetic component, and other factors such as a lack of fiber or fruit and vegetables in the diet. It has been estimated that the traditional risk factors such as elevated plasma cholesterol and diabetes explain about 50% of the genetic component. However, all of these risk factors combined may account for only two-thirds of the incidence of atherosclerosis. The possible cause of atherosclerosis unrelated to these risk factors is the subject of ongoing investigations and many theories have been postulated. The focus of the research in this thesis is on two risk factors, high cholesterol, or more precisely LDL cholesterol that has been oxidatively modified, and diabetes mellitus.

High Cholesterol

The first risk factor for atherosclerosis that was identified was high total cholesterol. More than 100 genes have been identified related to the synthesis, regulation, and transport of cholesterol in humans. This is due to the important role that cholesterol plays in many life sustaining processes such as cellular membrane

physiology, dietary nutrient absorption, reproductive biology via steroid hormones, stress responses, salt and water balance and calcium metabolism [32]. It therefore is a striking paradox that this important life sustaining substance has been found to be responsible for the leading cause of death in the industrialized world.

Elevated total serum cholesterol is unique among all the other risk factors in its ability to promote the development of lesions in the absence of any of the other risk factors. Humans with low plasma cholesterol levels have lower levels of atherosclerotic disease even when other risk factors, such as smoking and hypertension, are present. It has been demonstrated that a 1% decrease in plasma total cholesterol concentrations is associated with a 2% decrease in the incidence of coronary artery disease over a period of 5-8 years [33]. Five major statin trials have provided conclusive evidence of the benefits of reducing cholesterol concentrations [34-38]. Risk factors, other than increased plasma cholesterol, can accelerate the atherosclerotic process, but in the absence of dyslipidemia they contribute little to atherogenesis [39]. In addition, there is no known natural mammalian animal model exhibiting human-like atherosclerosis in which atherosclerosis can be induced without altering cholesterol profiles [40]. All of these facts support the role of cholesterol as a causative factor in the development of atherosclerosis and therefore, reducing cholesterol with cholesterol lowering drugs, such as the statins, remains the principle means of reducing atherosclerotic disease.

The lipoproteins that transport cholesterol also play an important role in atherosclerosis. Lipoproteins are involved in the task of transporting dietary and

endogenously produced lipids to where they are needed in the body. Elevated LDL, the major carrier of cholesterol, is a well established risk factor for atherogenesis [41-43] and of all the lipoproteins its role in the development of atherosclerosis has received the most attention.

Lipoproteins are spherical particles with lipophilic cores containing varying amounts of cholesteryl esters and triglycerides. The core is surrounded by a monolayer of phospholipids. Also around the core is one or more long chain polypeptide(s) called an apoprotein (apo) that enables the transport of core lipids within the aqueous environment of the blood and tissue. Chylomicrons transport exogenous dietary lipids. Very low-density lipoprotein (VLDL), LDL, and high-density lipoprotein (HDL) transport endogenous lipids made mainly by the liver.

VLDL synthesized by the liver contains high levels of triglyceride fatty acids. On the surface of VLDL there are two apoproteins, apoB-100 and apoE enabling the transport of these triglyceride fatty acids either to muscles to be used for energy or to adipose tissue to be stored for future use. After releasing these triglyceride fatty acids, the remaining VLDL particle is metabolized to a smaller cholesterol-rich lipoprotein, LDL, by further removal of triglycerides and a dissociation of apoE.

LDL is the major carrier of cholesterol to the cells of the body. The core of LDL is high in cholesterol but also contains fatty acids and some lipid soluble antioxidants such as a-tocopherol (vitamin E). ApoB-100, the remaining apoprotein

from VLDL, is the only apoprotein on LDL. LDLs principle atherogenic mechanism is its major role in supplying cholesterol to tissues.

In contrast to VLDL and LDL, HDL is athero-protective [44, 45]. The core of HDL contains less cholesterol than LDL and much less triglyceride than VLDL. ApoA-1 is the predominant apoprotein on HDL. HDL's principle athero-protective mechanism is its major role in reverse cholesterol transport (RCT), the transportation of cholesterol from the peripheral tissues back to the liver. In mice injected with cholesterol, the rate of cholesterol clearance was highly correlated with the level of HDL providing evidence that RCT contributes greatly towards decreasing the risk of atherosclerotic lesion formation [46].

Excess cellular cholesterol has harmful effects on the cell membrane. Some of these effects include a loss of fluidity leading to the dysfunction of integral membrane proteins and receptors and a disruption of membrane domains causing a disruption of normal signaling events [32]. The accumulation of cholesterol in arterial cells can occur via three mechanisms – the endogenous synthesis of cholesterol by the cells themselves, the uptake of cholesterol-rich lipoproteins such as LDL via receptors on the cell surface, or by receptor independent insudation. Multiple pathways have evolved to protect cells against excess cholesterol and the harmful effects of its accumulation [47].

Cholesterol levels in the cells regulate the production of endogenous cholesterol by the cell via a feedback mechanism. When the cholesterol levels in the cytoplasm are low, sterol regulatory element-binding pProtein – 1 (SREBP-1), a 125 kDa transcription factor that is attached to the nuclear envelop and endoplasmic

reticulum, [48, 49] is cleaved and activated. SREBP-1 can then enter the nucleus, where it binds and activates the transcription of genes that are involved in the making of endogenous cholesterol. Three-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme required for the synthesis of endogenous cholesterol, is one of the genes upregulated. The increased production of HMG-CoA results in an increased production of endogenous cholesterol by the cell. An elevated level of cholesterol in the cell inhibits HMG-CoA reductase leading to a decreased production of endogenous cholesterol. The down regulation of endogenous cholesterol synthesis is one way in which the body attempts to prevent excess cellular cholesterol accumulation.

Cholesterol levels in the cytoplasm are also capable of regulating the expression of the low density lipoprotein receptor (LDL-R), a cell surface transmembrane protein, again through the action of SREBP-1 which activates the transcription of the LDL-R gene. The role of LDL-R in the regulation of the plasma cholesterol level has been well established [50, 51]. Upregulation of the LDL-R leads to the enhanced uptake of LDL from the plasma thereby lowering the circulating cholesterol levels and increasing cholesterol within the cell [52]. LDL is recognized by the cells via the attachment of the N-terminal domain of the apoB-100 molecule on the LDL surface to the LDL-R. Upon attachment to the receptor both the LDL and the receptor undergo endocytosis. Once inside the endosomes, the low pH causes the LDL particle and its receptor to dissociate. The receptor may then be recycled to the cell membrane where it may bind to additional LDL. Meanwhile the LDL particle is degraded in the lysosomes. The protein components

undergo hydrolysis to their individual amino acids and the lipid components undergo hydrolysis to free cholesterol. The hydrolyzed products may then exit the lysosome and enter the cytoplasm of the cell.

This receptor-mediated uptake of LDL observes saturation kinetics. When cholesterol levels reach the appropriate level in the cell the cleavage of SREBP-1 is inhibited. It can no longer enter the nucleus and activate the transcription of the LDL-R. As a consequence, LDL-Rs on the cells surface are down-regulated decreasing the removal of LDL from the plasma by this method and increasing plasma LDL levels. This receptor mediated process accounts for approximately 88% of the degradation of plasma LDL in mice, 73% in hamsters, and 72% in rabbits. The amount of plasma LDL degraded by the LDL receptor system can range from 33 - 66% in humans and averages around 58% in humans consuming diets relatively low in cholesterol content [53]. The increasing plasma LDL levels seen in humans as they age is generally due to a decrease in the number or upregulation of LDL-Rs resulting in a decreased capacity to remove LDL from the plasma [54, 55]. While the down-regulation of the LDL-R is another way in which the body attempts to prevent excess cellular cholesterol accumulation it can lead to an increase in plasma LDL levels and the adverse consequences associated with such as increase.

When plasma cholesterol levels are elevated, more LDL may enter through the endothelial cell gap junctions in a non-receptor mediated way. Usually the arterial EC barrier is selectively permeable. The glycocalyx, charge, and compactness of the endothelial basement membrane contribute to its selectivity

toward molecules based on charge and size [56]. The single contiguous layer of ECs lining the arteries has both tight and gap junctions and the space between the gap junctions is about 260 Angstroms. The average size of an LDL particle is also 260 Angstroms but ranges anywhere from 200 to 300 Angstroms. Particles less than 258 Angstroms can easily pass through the gap junctions. A decrease of only 5% in the diameter of LDL can result in a 50% increased rate of LDL uptake [57, 58] through normal gap junctions.

Under normal plasma cholesterol levels passage through endothelial cell gap junctions usually accounts for approximately 42% of the LDL plasma clearance in humans with normal LDL-R activity. In those that lack LDL receptor activity it is responsible for 100% of the LDL clearance that takes place [53, 59-62]. Unlike the receptor dependent process, which occurs primarily in the liver, 60 – 70% of the receptor-independent transport activity occurs in extrahepatic tissue [53, 59, 60]. This non-receptor mediated uptake of LDL observes linear uptake kinetics, not saturation kinetics, in response to the plasma LDL concentration [63]. LDL particles are therefore able to cross the endothelium and enter the intimal space in direct proportion to their serum concentration.

Elevated LDL probably plays an even bigger role in initiating and perpetuating atherosclerosis than was initially believed [13] because it is now known that it is not only the amount of LDL but also particle size and oxidative state that is important in the development of atherosclerosis. Smaller and/or oxidized LDL particles have been shown to be more atherogenic than native, normal sized LDL particles. Since smaller LDL particles are more atherogenic but

contain less cholesterol than the larger LDL particles measurement of LDL cholesterol may not be the most accurate indicator of atherogenic potential. A more accurate measurement may be the determination of the number of LDL particles. Since there is only one apoB molecule on each LDL particle, the number of particles can be determined by determining the number of apoB molecules. However, there is also one apoB molecule on each VLDL particle and every VLDL particle ends up as an LDL particle. The assumption therefore might be that there would be a 1:1 ratio between these two particles but the biologic half-life of an LDL particle is at least nine times longer than that of a VLDL particle [64] so there is generally a ratio of nine LDL particles to every one VLDL particle in the blood. Measuring the number of plasma apoB particles, 90% of which is LDL associated, [65] is therefore a better method in determining LDL atherogenicity than measuring LDL cholesterol totals.

Endothelial dysfunction also results in an increase in permeability and allows more of the small, dense LDL particles to enter. There are several theories on how the endothelial cell barrier may become more permeable. The concept that is most widely accepted is that EC contraction induced by histamine, a vasoactive agent, causes the increased extravasation of macromolecules including lipids [56]. More recently it has been determined that histamine may reduce tight-junction protein expression causing leaky junctions [66]. In addition, vascular endothelial growth factor (VEGF) makes the endothelial monolayer leaky [67-69]and focal leaky areas are often associated with EC division [70].

The "response to injury" hypothesis originally proposed that endothelial denudation was the first step in atherosclerosis [71, 72]. However, most atherosclerotic lesions show no evidence of endothelial denudation so this theory was later revised to emphasize endothelial dysfunction or "injury" rather than denudation [73]. The response to injury hypothesis de-emphasizes the role of LDL in initiating atherogenesis and proposes that the initial event in atherosclerosis is an insult to endothelial and smooth muscles cells [73-75]. One study has demonstrated that endothelial dysfunction occurs following the ingestion of a high-fat meal but not after a low fat meal [76] and others have demonstrated that hypercholesterolemia causes focal activation of the endothelium in large and medium size arteries in humans and animals [77, 78]. Thus, it appears that increased plasma LDL itself may be responsible for the "injury" or dysfunction of ECs.

Once inside the intima, LDL particles aggregate. This aggregation of LDL particles tends to make it more atherogenic as demonstrated in a recent study in which the prevention of aggregation of LDL prohibited the formation of atherosclerotic lesions even in the presence of excess LDL [77, 79, 80].

Aggregation can be caused by the interaction between the LDL and the proteoglycans in the intima. Proteoglycans are macromolecules composed of a core protein and complex, long side-chain carbohydrates called glucosaminoglycans (GAGs). Aggregation of LDL has been shown to result primarily from direct ionic interactions between positively charged residues on the apoB molecule and negatively charges moieties of the proteoglycans. The interaction can also be

indirect, via bridging molecules such as lipoprotein lipase (LPL) [81] secreted by macrophages [82, 83]. Depending on its location LPL may have an opposing effect. When present on the endothelium it acts on circulating triglyceride lipoproteins in an anti-atherogenic manner but when present in the intima it enhances LDL and proteoglycan binding thereby increasing the proatherogenic effects associated with LDL aggregation [84].

The retained LDL can further induce its own aggregation [85]. Aggregation can also be induced by the enzymatic lipolysis of LDL by secretory phospholipase A2 [86] or by secretory sphingomyelinase (sSMASE) [87, 88]. In a study using mice expressing LDL defective in proteoglycan binding as compared to wild-type mice, Boren et al [80] demonstrated that the mice expressing the LDL defective in proteglycan binding developed less atherosclerosis than the wild-type mice. This supports the importance of proteoglycan binding as an early event in atherosclerosis.

There is some evidence that increased permeability at the sites of lesions does not fully explain the extent of lipid accumulation. The accumulation of lipids may also occur as a defect in lipid egress [89-92]. Once the LDL particles aggregate they may no longer exit the cell but become entrapped within the cytoplasm. Entrapment of LDL particles due to a change in their physical characteristics as a cause for atherogenesis is the foundation of two somewhat similar hypotheses of atherogenesis. One of these is the "response to retention" hypothesis and the other is the "oxidized LDL" hypothesis. The response to retention hypothesis proposes that the initiating event in atherosclerosis is the

retention and accumulation of LDL and other lipoproteins [93]. The basis of the oxidized LDL hypothesis is that the LDL retained is oxidized and that it is the oxidation that may be the initiating event in atherosclerosis.

Oxidized LDL

While elevated levels of LDL were initially associated with the risk for atherosclerosis, *in vitro* studies suggested that LDL itself was not atherogenic [51, 94]. In fact, incubation of LDL with normal macrophages possessing normal LDLRs did not support foam cell formation. The idea that the modification of LDL increases its atherogenic potential is just a little over 20 years old [95-97]. Brown and Goldstein first observed that chemical modification of LDL, in the form of acetylation, led to foam cell formation when incubated with macrophages. In 1979, Hessler and colleagues [98] demonstrated that LDL that had been oxidized caused injury to cells *in vitro* and speculated that oxidized LDL (oxLDL) might be important in atherogenesis. It was speculated, however, that the presence of anti-oxidants would prevent the oxidation of LDL *in vivo*.

But more recently the occurrence of LDL oxidation *in vivo* has been supported by several pieces of evidence. Oxidized apo B-100 epitopes and increased levels of lipid peroxidation products have been detected in the LDL extracted from both rabbit and human atherosclerotic lesions [99]. Immuno-histochemical staining of atherosclerotic lesions with specific monoclonal antibodies also has demonstrated the presence of oxLDL [100]. In addition, circulating anti-oxLDL antibodies have been found in human plasma and the levels of this anti-oxLDL correlate with the progression of atherosclerotic lesions

[101]. Also there is epidemiological evidence, including case-control and prospective cohort studies, indicating that low antioxidant consumption is associated with an increased risk of CVD [102-104]. Finally, studies in different animal models of atherosclerosis strongly suggest that progression of lesions can be delayed by intervention with several different antioxidants such as probucol, vitamin E, diphenylphenylene-diamine (DPPD), and butylated hydroxytoluene (BHT) [105-120]. In order to demonstrate that lesion reduction was due to the antioxidant activity of probucol and not its cholesterol lowering properties Carew et al., [121] treated one group of rabbits with probucol and another with lovastatin, an HMG-CoA reductase inhibitor that lowers cholesterol without antioxidant protection. Both groups had lower total cholesterol but only the probucol group had a reduction in the extent of atherosclerosis thereby demonstrating that the effect was due to probucol's antioxidant not its lipid lowering activity.

While LDL may undergo other modifications, including chemical, glycation, hydrolysis, and immune complex formation, oxidation is the most likely in-vivo modification of LDL in the arterial wall [94, 122-124] and the one most closely associated with atherosclerosis [125]. However, there is still no confirmation that there is an absolute requirement for LDL oxidation to initiate atherogenesis.

Oxidation of LDL is caused by a wide variety of ROS. ROS are largely produced by the arterial cells themselves as either by-products of their normal role or as a specific response to inflammatory stimuli. All three cell types involved in

atherosclerotic lesion development, ECs, SMCs and macrophages, are capable of contributing to the oxidation of LDL.

The term free radical has been equated with ROS, or oxidants, but by definition a radical is a molecule possessing an unpaired electron. However, with the exception of the hydroxyl radical most other free radicals are not strong oxidants. ROS such as hydrogen peroxide, superoxide, and the hydroxyl radical are products of normal oxygen metabolism. They are generated as a result of energy production from the mitochondria via the electron transport chain and as part of the respiratory burst utilized by white blood cells to destroy invading microorganisms. LDL may be modified by any enzyme system that can generate free radicals which includes myeloperoxidase, lipoxygenase, phospholipase and sphingomyelinase. All of these enzymes are present within arterial wall cells.

The oxidation hypothesis emphasizes the significance of the oxidative modification of LDL and the subsequent responses [126]. Oxidation of LDL occurs primarily in the intimal microdomains where, as previously discussed, it may be bound to proteoglycans and aggregated [127]. Oxidation of LDL is influenced by extrinsic local factors such as pH, transition metal availability, the presence of specific enzyme systems, and local antioxidant concentrations. For example, an acidic environment will promote LDL oxidation by certain enzymes and may also effect the interactions of LDL with arterial wall components. Internal factors such as the antioxidant content and fatty acid composition of LDL also affect its susceptibility to oxidation.

All the particles in LDL are susceptible to oxidative modification. ROS react with all classes of biological molecules but the polyunsaturated fatty acids (PUFAs) are particularly susceptible because of their double bonds. A high proportion of PUFAs in the LDL particle make it more susceptible to oxidation whereas a high proportion of monounsaturated fatty acids (MUFAs) protects against oxidation [128]. The molar ratio of PUFA to antioxidant in the LDL particle is also important in determining its susceptibility to oxidation with a higher ratio increasing susceptibility [129, 130]. In addition, small dense LDL particles are more susceptible to oxidation than large less dense particles [131, 132] due to their lower levels of alpha-tocopherol and ubiquinol-10, two important antioxidants.

Known as lipid peroxidation, the oxidation of PUFAs in LDL is particularly damaging because it proceeds as a self-perpetuating chain-reaction. In lipid peroxidation a free radical, such as the hydroxyl radical (OH), abstracts a hydrogen atom from a PUFA double bond. An unstable carbon centered lipid radical is produced (L·). Because of its instability it undergoes a molecular rearrangement resulting in a more stable conjugated diene. But even the more stable conjugated diene reacts very quickly with molecular oxygen forming a lipid peroxyl radical (LOO·). The lipid peroxyl radical further propagates the peroxidation chain reaction by abstracting a hydrogen atom from a nearby PUFA. The resulting lipid hydroperoxide (LOOH) can easily decompose to form a lipid alkoxyl radical (LO·). These decomposed lipid hydroperoxides can undergo beta-scission reactions forming reactive aldehyde compounds such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE). Since oxidants themselves are very short lived most

studies focus on the products of oxidation reactions, such as MDA and 4-HNE, which readily combine with functional groups on proteins, lipoproteins, and DNA potentially altering their behavior. Alpha tocopherol can break the chain reaction by forming a tocopherol radical. While this is still a radical species it is one that has very low reactivity. The tocopherol radical can be reduced by co-antioxidants such as ascorbate (vitamin C) and be exported out of the lipoprotein particle into the aqueous phase thus leading to chain termination as opposed to chain propagation. HDL also contains an enzyme called paroxonase, which has been shown to protect LDL from oxidation and prevent accumulation of lipid peroxidation products on LDL. This antioxidant effect of HDL is another method, along with its role in RCT, by which it is athero-protective.

Once produced, reactive aldehydes can diffuse and cause cytotoxic and mutagenic damage [133, 134]. They also readily bind to lysine groups in the apoB-100 protein molecule, forming Schiff bases and altering the surface charge of LDL. Oxidative modification of proteins may result in cross-linking, peptide fragmentation, or in the conversion of one amino acid to another. These modifications may alter the secondary and tertiary structure of the protein and in doing so may subject previously unexposed regions to further oxidation.

As much as 1% of the oxygen consumed by the average human may oxidatively modify proteins. In an elderly person 30 – 50% of their total cellular protein may be oxidatively modified [135] because there is less protection against oxidative modification in old cells and tissues than in young ones. LDL lipid peroxidation does not represent the only manifestation of oxidative stress that may

be important in atherosclerosis. Recent research indicates that other oxidative events may be present and could play a role in atherogenesis including the nonenzymatic oxidation of the phospholipids in LDL particularly those containing arachidonic acid at the sn-2 position [136]. Comprehensive reviews of the oxidative modification hypothesis of atherosclerosis have been published [137-139].

LDL that is oxidized is not a single, well-defined entity. Its structural and physical properties vary according to the degree of oxidation [140]. The degree of oxidation is affected by the type of oxidant and the length of exposure to the oxidizing environment. Native LDL itself is heterogeneous and therefore a homogeneous product from oxidation would be impossible, particularly since there are many different components of the LDL particle such as cholesterol, phospholipids, cholesteryl esters, triglycerides and the protein portion that all may be oxidized to varying degrees [141]. The degree of modification can range from "minimal" (mmLDL), where the LDL particle is still recognized by the native LDL receptors [142], to extensively oxidized LDL (oxLDL), where the LDL particle is no longer recognized by the LDL receptor.

The biological effects of oxLDL also vary according to its degree of oxidation and the component that is oxidized. Evidence suggests that most of the atherogenic effects of oxLDL are derived from oxidized lipid components. The induction of adhesion molecules at the endothelial cell surface, such as VCAM-1 and ICAM-1 for the adhesion of monocytes, is affected by oxidized fatty acids and oxidized phospholipids, such as lysophosphatidylcholine (LPC). LPC is also

chemotactic to both monocytes and T-cells by inducing MCP-1. All three stages of monocyte binding: tethering via selectins, activation, and attachment are effected. OxLDL is also chemostatic for tissue macrophages. This could contribute to the trapping of macrophages once they have entered the subendothelial space. Oxidized fatty acids also increase the proliferation of arterial SMCs and highly oxidized LDL can inhibit endothelial cell migration which may hinder the repair of ulcerated plaques in advanced lesions. OxLDL has also been shown to be cytotoxic to macrophages and endothelial cells. This may contribute to an amplification of the inflammatory process and the formation of the necrotic core found in advanced lesions. Oxidized LDL may also directly alter the vasomotor properties of coronary arteries by inhibiting the ability of endothelial cells to release endothelium-derived relaxing factor or nitric oxide (NO). In addition to these biological effects, oxidized lipids and oxidants also appear to activate matrix metalloproteinases (MMPs) which might play a role in advanced plaque instability and rupture. They also promote pro-coagulatory responses from both the endothelium and platelets and have many other atherogenic effects.

Diabetes mellitus

In contrast to the decrease in overall death rates for CVD, death rates for diabetes have been gradually increasing since 1986. Diabetes was the sixth leading cause of death in 2000, however, this is probably under reported as only 35 – 40% of diabetics have the disease listed anywhere on their death certificate and only 10 – 15% have it listed as an underlying cause of death. Diabetics are also two to five times more likely to develop CVD than non-diabetics [143, 144]. In people with

diabetes, risk factors for CVD include elevated fasting plasma glucose, elevated blood pressure, elevated cholesterol and triglycerides and obesity. These partly explain the 60- 70% of deaths caused by CVD in people with diabetes [7].

Diabetes mellitus is a syndrome initially characterized by a loss of glucose homeostasis. Normally glucose levels are elevated in the blood following a meal. In response, insulin, the hormone that allows glucose to enter the cells of the body, is secreted from the beta cells of the pancreas. Type I diabetics have a complete lack of insulin production due to the destruction of their beta cells. Type I diabetes exists in two forms; immune mediated or an idiopathic form. Regardless of the cause, the beta cells of the pancreas are destroyed and little or no insulin is produced. At diagnosis, 50% of type I diabetics have antibodies against insulin and/or antibodies against islet cells of the pancreas. It is estimated that 5 – 10% of Americans who are diagnosed with diabetes have type I diabetes. This type of diabetes generally has a rapid onset that occurs most frequently in juveniles and therefore type I diabetes was at one time referred to as juvenile onset diabetes. It also requires the administration of insulin and therefore has been referred to as insulin dependant diabetes. However, these two terms may no longer exclusively describe type I diabetes and therefore are no longer used.

The majority of diabetics, 90 – 95%, have type II diabetes mellitus. Type II diabetics are either insulin resistant (IR) or insulin deficient, depending on the stage and progression of the disease. The appearance of overt type II diabetes is typically preceded by a pre-diabetic period in which the patient displays insulin resistance and hyperinsulinemia but relative normoglycemia. In insulin resistance the cells of

the peripheral tissues, such as skeletal muscle, are not as sensitive to insulin and therefore require more insulin to take up glucose. In response to the cells being less sensitive to insulin the beta cells compensate by increasing insulin production leading to hyperinsulinemia. However, eventually the beta cells cannot secrete enough insulin to overcome insulin resistance. During this time the insulin levels remain elevated, often above normal levels, but not high enough to cause the peripheral cells to take up an adequate amount of glucose. Eventually the body cannot maintain these high levels of insulin production. The beta cells produce decreased insulin and hypoinsulinemia and hyperglycemia, the hallmarks of diabetes, develop.

From 1980 through 2004, the number of Americans with diagnosed diabetes more than double from 5.8 million to 14.7 million people. Including those with undiagnosed disease, diabetes mellitus currently affects approximately 18.2 million individuals in the United States; the majority of which is type II diabetes. Between 1983 to 2003 the prevalence of type II diabetes increased from 5.9% to 13.8% [145]. It has been predicted that by the year 2050, 29 million American will be diagnosed with diabetes which will be 165% more than in the year 2001 [146].

Of great concern is the growing number of children and adolescents now being diagnosed with type II diabetes related to obesity [3]. The link between obesity and type II diabetes has caused some to suggest using the term "diabesity" to better describe the current epidemic. Developing type II diabetes rises with increasing body weight, and is about 5-10 times greater in those having a body mass index of 30 or above than in those with a body mass index below 25. Those with a

body mass index of 30 or above are classified as obese [4, 9]. There are believed to be more than 250 million obese adults worldwide.

This does not bode well for the future. Currently an estimated 46 to 49 million adults in the US may have insulin resistance and it is predicted that one in four of them will develop type II diabetes [147]. Diabetes is predicted to reach epidemic proportions not only in the United States but throughout the world. The World Health Organization (WHO) predicts that the number of people with diabetes will double within just one generation, from 150 million in 2001 to an estimated 300 million in 2025. The developing world is expected to have the greatest increase in diabetics. In developed countries diabetes is predicted to rise by a total of 41% between 1995 and 2025 while in developing countries it is predicted to rise by 170 within the same time period [148].

The cause of diabetes type II is not fully understood and is linked with not only obesity as discussed above, but also the lack of exercise, and hyperlipidemia. Obesity is associated with a low-grade state of inflammation, probably as a consequence of the secretion of pro-inflammatory cytokines by adipocytes that may underlie the insulin resistance [149]. Early in 2001, researchers identified a hormone called resistin which might explain the obesity-diabetes link at a biochemical level [150]. Resistin more recently has been determined to have a potential role in atherogenesis [151].

Two possible mechanisms responsible for insulin resistance include the activation of protein kinase C (PKC) [152] and elevated TNF-α [153]. Both of these mechanisms affect the insulin receptor by deactivating it and interfering with

glucose uptake into the cell [154]. The insulin receptor is composed of two subunits which extend through the cell membrane. When insulin binds to the subunits, it turns on the enzyme tyrosine kinase which causes a series of phosphorylations to occur on the tyrosine resides. The phosphorylated tyrosine activates second messengers causing the translocation of glucose into the cell [155]. PKC in the cell is activated when there are elevated free fatty acids. The activated PKC causes the phosphorylation of serine and threonine residues on the insulin receptor, not tyrosine. When this occurs glucose is not translocated from the blood stream and into the cell via the insulin receptor. TNF-α secretion increases due to weight gain and also negatively affects the insulin receptor [156]. It stimulates phosphatases in the cell to take off the phosphate groups on the tyrosine residues which again inhibits the translocation of glucose into the cells leading to hyperglycemia [157, 158].

The diabetic's lipoprotein profile is characterized by changes in lipoprotein concentrations and composition [159]. On average, the plasma total cholesterol of diabetics is similar to non-diabetics. However, type II diabetes is associated with a dyslipidemia that includes increases in plasma LDL, small dense LDL, VLDL, lipoprotein(a), and a decrease in HDL [160]. This creates and even more atherogenic lipoprotein profile in the diabetic.

Patients with insulin resistance and type II diabetes also characteristically have elevated postprandial triglyceride-rich lipoproteins compared to non-diabetics. Fasting hypertriglyceridemia levels have been demonstrated to predict this abnormal postprandial response to a dietary fat load. A strong correlation exists

between plasma insulin and the postprandial TG response to a fat meal, as well as to the postprandial levels of large VLDLs and chylomicron (CM) remnants. In the fasting state, increased plasma insulin, a marker of insulin resistance, is also related to increased fasting plasma levels of large VLDL and CM remnants [161-168].

Diabetes, types I and II, have been shown to significantly increase the extent and clinical complications of atherosclerosis [169, 170]. Initially the view was that many of the pathologic effects of diabetes were from the condition of hyperglycemia itself. However, it was noted that increased lesion development was already apparent upon diagnosis of diabetes indicating that these lesions were most likely developing during the euglycemic pre-diabetic state. Clinical trials have documented that a reduction in LDL cholesterol significantly reduces the risk of CVD in diabetics while a reduction of blood glucose levels has a relatively small effect on atherosclerosis [170].

Since the oxidative modification of LDL is an important initiator of atherosclerosis it was important to determine whether an increase in oxLDL was present in diabetics. Oxidative stress has been demonstrated to be elevated in diabetics [171] as indicated by decreased plasma levels of vitamins E and C, and increased serum MDA [172] and urinary F2-isoprostanes [173]. Fatty acid hydroperoxides, another indicator of oxidative stress, are elevated 2 to 3 fold. Diabetics also have elevated levels of autoantibodies to oxLDL [174] and the resulting immune complexes between oxLDL molecules and their autoantibodies are more readily taken up by macrophages causing the accumulation of cholesteryl esters and an increase in the release of Il-1 and TNF-α. In addition, the LDL of

diabetics has a higher affinity for proteoglycans *ex vivo* [175, 176] and appears to be more prone to oxidative modification than that of non-diabetics [177]. The source of free radicals in diabetes is not completely understood but cells may produce increased superoxide when exposed to glucose and there may be diabetes associated mitochondrial dysfunction increasing mitochondrial generation of ROS. Elevated glucose has also been shown to increase the expression and activity of 12-lipoxygenase in SMCs [178]. Therefore, while the greater risk for and development of atherosclerosis in diabetics could be due to a number of factors including dyslipidemia, hyperglycemia, and insulin resistance, increased oxidative stress appears to play an important role.

One of the effects of increased oxidative stress may be to exacerbate another process known as glycation. Chronic exposure of proteins to increased glucose concentrations in diabetics leads to the formation of advanced glycation end-products (AGEs) [179]. Oxidation and nonenzymatic glycation are mutually reinforcing processes leading to glycoxidation. All three processes, glycation, glycoxidation, and oxidation are complex interrelated processes. For example, glycated LDLs are more oxidizable than nonglycated LDLs and increased lipid oxidation appears to be sufficient to cause AGE formation in euglycemic animals [180]. Carboxymethyl lysine (CML), an irreversible advanced stage molecular structure generated through glycoxidation, [181] is increased in the atherosclerotic lesions of diabetics. One study demonstrated that the development of atherosclerosis is synergistically promoted by glycoxidation and lipid peroxidation

of LDL [182]. This accelerated atherosclerosis is a major cause of morbidity and mortality in diabetics.

CHAPTER III

SCAVENGER RECEPTORS

Since it was determined that oxLDL was the ultimate source of cholesterol that accumulated in developing foam cells, investigators initially thought that foam cell formation was mediated by the LDL-R. However, patients without functional LDL-Rs had early atherosclerosis with foam cells similar to those that developed in people with functional LDL-Rs, demonstrating that functional LDL-Rs were not required for foam cell formation. In addition, macrophages completely lacking LDL-Rs could become foam cells. The uptake of oxLDL by macrophages was eventually demonstrated to take place by way of a "scavenger receptor" [183]. Unlike the LDL-R, scavenger receptors (SRs) do not down regulate in the presence of high LDL concentrations. Macrophages may therefore continue to take up oxidized LDL until they become engorged with stored cholesterol.

The expression of SRs is modulated by various growth factors, inflammatory cytokines, chemokines, lipids, cholesterol and modified lipoproteins, all of which are present within the atherosclerotic lesion [184]. SRs were originally implicated in the pathological deposition of cholesterol in the foam cells of atherosclerotic lesions through receptor-mediated uptake of modified LDL. However, other functions of SRs, including endocytosis, phagocytosis, adhesion, and signal transduction triggered by the binding and

uptake of modified LDL, have subsequently been determined to also be involved in the development of atherosclerosis.

There are six different classes of SRs. All are similar to the LDL-R in that they are cell surface transmembrane receptors. The name scavenger receptor was well chosen, because SRs recognize a spectrum of structurally unrelated ligands and although each scavenger receptor shows a broad specificity that often overlaps that of another scavenger receptor, a clear preference for certain ligands by each receptor is apparent [184]. While the ligands are themselves unrelated they all share one similarity in that they are all negatively charged macromolecules. OxLDL is one such negatively charged macromolecule. Other modified lipoproteins recognized by scavenger receptors, include acetylated LDL, glycoaldehyde, which is an AGE-modified LDL, and nitrate-modified LDL. SRs also bind other ligands such as thrombospondin, collagen, phosphatidylserine, liposomes, bacterial components such as endotoxin, and apoptotic cells [184]. *In vivo*, oxLDL binding and uptake by macrophages appears to be the most relevant for the atherosclerotic process.

SRs also participate in the recognition and clearance of apoptotic cells and bacteria [185]. Removal of dead or apoptotic cells is important both for embryological development and for maintaining tissue homeostasis. The recognition of oxLDL by SRs may be explained by the structural similarities between cell membranes and lipoproteins. Both consist of phospholipids, cholesterol, and (glyco)protein. Oxidative damage to a lipoprotein particle may create epitopes that resemble the epitopes expressed by cells that undergo

apoptosis or by senescent red blood cells [186]. Thus, macrophages are responding in a protective manner by clearing the tissues of dead cells.

The first scavenger receptor was discovered on macrophages and was called scavenger receptor A (SR-A). The molecular characterization of SR-A took place in 1990 when it was finally cloned [187]. In recent years, several new members of the scavenger receptor family, such as CD36, SRB1, and CD68 have been discovered on macrophages and have also been cloned on the basis of their ability to recognize modified lipoproteins [188, 189].

The receptor focused on in this study was the first scavenger receptor originally identified as an endothelial cell scavenger receptor [190, 191]. Based on sequence data showing that the molecule has a long C-terminal extracellular domain belonging to the C-type lectin family, the molecule was termed LOX-1 for lectin-like oxLDL receptor-1 [191]. LOX-1 is a type II membrane protein classified as a class E scavenger receptor that has been demonstrated to act as a cell-surface receptor of oxLDL [192, 193]. It does not share any homology with any other of the known SRs. In addition to the extracellular C-type lectin domain, LOX-1 consists of a hydrophobic transmembrane domain and a cytoplasmic N-terminal domain. Although the calculated mass of this protein is about 31 - 32 kDa [192-194], it is found endogenously as a 40 kDa precursor form with N-linked high mannose carbohydrate chains and a 48 - 50 kDa mature form due to further glycosylation [193]. This is the result of post-translational modification of four potential N-linked glycosylation sites located on the C-terminal domain. The N-linked glycosylation of LOX-1 appears to be necessary for adequate

transportation to the cell surface and efficient ligand binding [193]. The lectin domain is the functional domain that recognizes LOX-1 ligands and the C-terminal end residues and several conserved positively charged residues spanning the lectin domain are essential for oxLDL binding. In the N-terminal cytoplasmic domain there are several potential phosphorylation sites. An unknown protease is able to cleave the N-terminal amino-acids at two cleavage sites, located in the membrane proximal extracellular domain, releasing soluble LOX-1 (sLOX-1) into the bloodstream. The LOX-1 receptor is not only present on the endothelium of vascular rich organs (liver, lung, brain, placenta), the aorta, coronary and carotid arteries, but also on macrophages and vascular SMCs in atherosclerotic lesions, including those of humans [193, 195].

It has been demonstrated that LOX-1 promotes the binding, internalization and degradation of oxLDL, [196], lysophosphatidylcholine, and oxidized fatty acids. The binding of oxLDL to LOX-1 may trigger the activation of the NF-κB signal transduction pathway [197]. Binding experiments have demonstrated that native LDL and acetylated LDL do not compete with LOX-1 for uptake of oxLDL [191, 196]. LOX-1 expression has been shown to be upregulated by minimally oxidized LDL but not extensively oxidized LDL. TNF-α [198], transforming growth factor beta (TGF-β) [194, 199], angiotensin II [200] and shear stress also upregulate LOX-1.

The binding of platelets to LOX-1 enhances the release of endothelin-1 from ECs inducing endothelial dysfunction and promoting the atherogenic process [201]. LOX-1 is also a key factor in oxLDL-mediated monocyte

adhesion to human coronary artery endothelial cells [202]. LOX-1 has the ability to capture leukocytes under physiologic shear stress suggesting that it functions as a vascular tethering ligand [203]. C-reactive protein (CRP) also enhances endothelial LOX-1 expression [204] and increases both human monocyte adhesion to endothelial cells and oxLDL uptake by these cells. Heparin-binding epidermal growth factor-like growth factor (HB-EGF), a potent mitogen for vascular SMCs, also induces LOX-1 expression in cultured bovine aortic SMCs [205]. Homocysteine, an atherogenic amino acid believed to exert its effects through oxidative stress, enhances endothelial LOX-1 gene expression in aortic ECs. The antioxidant N-acetylcysteine (NAC) suppresses this enhanced endothelial LOX-1 gene expression [200]. LOX-1 is also known to bind apoptotic cells and oxidized red blood cells.

OxLDL stimulated LOX-1 expression in human coronary artery ECs is downregulated by pre-treatment with statins [195, 206]. A high concentration of statins (10 uM) is more potent than a low concentration (1 uM) in downregulating LOX-1. LOX-1 on ECs induces reduction of nitric oxide (NO) release and up-regulation of adhesion molecules, thereby contributing to the development of atherosclerosis [207]. Antisense to LOX-1 mRNA decreases LOX-1 expression and the subsequent expression of the adhesion molecules, VCAM-1, ICAM-1 and the selectins [206]. Monoclonal antibodies against LOX-1 can inhibit oxidized LDL binding to cultured bovine aortic ECs by 50 to 75%. This indicates that LOX-1 may play an important role in the uptake of oxLDL from the bloodstream and the development of atherosclerotic lesions.

The presence of LOX-1 has been demonstrated in both animal models of atherosclerosis and human atherosclerosis. LOX-1 ligand levels have been shown to be significantly elevated in the plasma of Wantanabe heritable hyperlipidemic rabbits [208]. In addition, increased expression of LOX-1 has been demonstrated in the initial atherosclerotic lesions of Watanabe heritable hyperlipidemic rabbits primarily within the aortic intima at the earliest stages of lesion development and most prominently in the ECs of the lesions. However, even the ECs in the non-lesion areas exhibited distinctive LOX-1 immunoreactivity in these animals [209] demonstrating that the hyperlipidemia itself increased the LOX-1 expression. LOX-1 has also been shown to be significantly increased in diabetic rat aorta with the most immunoreactivity in ECs, particularly those in the bifurcations of artery branches from the aorta [210]. Studies have also shown that the monoctye-derived macrophages of humans with type II diabetes also over express LOX-1.

Diabetics, as discussed previously, have dyslipidemia but not necessarily hyperlipidemia. Even without hyperlipidemia, diabetics have markedly upregulated LOX-1 expression. LOX-1 has been shown to bind not only oxLDL in diabetics but also AGEs, the non-enzymatically glycosylated proteins which accumulate in the vascular tissues of the aging and diabetic populations [211].

In cultured aortic ECs, streptozotocin-induced diabetic rat serum and AGE-bovine serum albumin (BSA) induced LOX-1 expression, while control rat serum with only high glucose did not. This suggests that glucose alone is not responsible for the induction of LOX-1 expression on ECs but that plasma proteins that have undergone glycosylation are the more likely culprits [212]. In

contrast, *in vitro* studies using human monocyte-derived macrophages demonstrated that incubation with glucose would enhance both LOX-1 gene and protein expression in a dose- and time-dependent manner. Antioxidants, PKC, mitogen activated protein kinases (MAPKs), nuclear factor-kappaB (NF-κB), and activated protein-1 (AP-1) inhibitors all were able to abolish the induction of LOX-1 gene expression in macrophages by glucose. [213]. Whether it is glucose alone that increases LOX-1 expression, as seen in human monocyte-derived macrophages, or whether it takes AGE-modified plasma proteins to increase LOX-1 expression as seen in cultured aortic ECs, both processes are indicative of the relevance of the study of LOX-1 in relation to diabetic atherosclerosis.

CHAPTER IV

THE SYRIAN F_1B HAMSTER

Animal models of human diseases are often used in experimental studies because environmental and genetic backgrounds can be strictly controlled in animal models. This control is difficult when studying human disease in humans as environmental and genetic backgrounds differ from individual to individual and population to population. Recently much of the animal research has used gene "knockouts" where targeted genes are deleted from the animals. However, scientists must be cautious in their interpretation of results from gene knockout animals since gene deletions may have secondary phenotypic effects and are not tissue specific. Additionally, gene deletions may cause compensatory changes in the expression of other genes in these animals and this may also confound the results.

The Syrian hamster, *Mesocricetus auratus,* has been used extensively as an experimental animal model since 1930 when hamsters captured in Syria where bred at Hebrew University. The normal life span of the Syrian hamster is up to 3 years. While the hamster is classified as an omnivore and is capable of predation, some of the structural features of its gastrointestinal tract and some features of its feeding behavior are more similar to that of an herbivore [214]. Hamsters have a rigid and regular two hour feeding schedule, feeding 12 times throughout the day and night in a 24 hour period. They will normally consume about 2.5 calories/100g at each 2 hour interval when fed ad libitum or about 30 calories/100g per day for a sedentary hamster. Hamsters have

limited physiologic ability to withstand food deprivation. Their basic response to a shortage of food is to reduce their energy expenditure rather than increase their food consumption when food again becomes available. Fasting for as short as 24 hours may induce starvation anorexia or may decimate the bacteria and protozoa in the forestomach and cecum necessary for digestion. Weight loss greater than 22% may cause hypothermia, unconsciousness and whole-body tremors [215, 216].

Lipoprotein Metabolism

Hamsters have long been used as an animal model for the study of lipoprotein metabolism [217] since their lipoprotein metabolism has many attributes in common with humans [218, 219]. Hamsters have relatively high serum LDL concentration when compared to mice and rats [220]. In the hamster, as in humans there are appreciable levels of LDL as well as HDL [221]. And although unlike humans, HDL is the prominent lipoprotein present in hamster plasma, at least one third of the cholesterol is associated with the LDL fraction under normal dietary conditions [222]. The apoprotein B concentration is sevenfold greater in hamsters than in rats. In rats, 47% of apoprotein B is associated with LDL, while in hamsters, 70% of apoprotein B is associated with LDL [223, 224]. In general the apoprotein profile of the hamster has many similarities to that of humans. However, there is a difference in apoE expression between humans and hamsters. ApoE is found in the LDL fractions of hamsters but very little is found in the LDL fractions of humans. ApoE is also a more prominent constituent of VLDL in hamsters than in humans. Overall the plasma lipoprotein profile of humans in more analogous to hamsters than to mice or rats [225].

Unlike livers from rat or mouse, the liver of the Syrian hamster secretes only apoB-100 containing VLDL, as is the case in humans [226]. Apoprotein B-48 containing lipoproteins are of intestinal origin only [227] in hamsters and in humans. This tissue-specific expression of apoB-100 by the liver only and apoB-48 by the intestine only is a distinct advantage of the hamster model [227, 228] over the mouse and rat model. Hamsters have also been shown to express plasma cholesteryl ester transfer protein (CETP) activity which is regulated by the level of cholesterol-containing lipoproteins in plasma as is the case in humans [229, 230]. Rats do not express CETP activity [231].

The hamster LDL-R receptor gene has been isolated and characterized and shows strong sequence and structural similarities to the human gene [232]. The receptor-dependent transport of LDL in the hamster is also similar to humans. In the hamster, the liver is the major site for the removal of plasma LDL, accounting for 73% of the LDL degradation. This is due to both the high rate of receptor-mediated transport per gram of tissue and the large size of the liver. Most of the LDL degradation is mediated by the LDL-R following saturation kinetics.

In human lipoprotein metabolism, the concentration of circulating LDL is determined by the rate of LDL formation relative to the rate of receptor dependent LDL removal from the plasma [233]. When dietary intake of cholesterol and triacylglycerol is minimal and little lipid is reaching the liver in the chylomicron remnant, the rate at which LDL cholesterol is being produced and entering the plasma is low relative to the rate at which LDL can be removed from the plasma by receptor-dependent transport. This is also true for the hamster where the LDL production rate is only about 150 ug/h while the maximal achievable rate of receptor dependent removal is about 700 ug/h [234]. In the

hamster, as in humans, the liver has a relatively low rate of cholesterol synthesis. The extensive work of Dietschy, Spady and co-workers has shown that the level of hepatic cholesterol synthesis in hamsters is equivalent to that found in humans, approximately 9 – 10 mg cholesterol/day/kg body weight [234, 235]. Rats and mice synthesize approximately 150 mg cholesterol/day/kg body weight, another reason why they are not good models of human lipoprotein metabolism [236].

The extrahepatic tissues of the hamster however, have high rates of cholesterol synthesis and may synthesize most of the cholesterol they need. Therefore, most of the apoprotein B-100 that is secreted from the liver in VLDL is returned to the liver carried either in VLDL remnants or in LDL. Relatively little is delivered to the peripheral organs [237]. Other animal models in which the rate of cholesterol synthesis in the liver is low compared to cholesterol synthesis in the whole animal are guinea pigs, pigs and several species of monkeys and baboons [236]. However, these species are not as easy to handle or as inexpensive as the hamster. They also have other dissimilarities to human lipoprotein metabolism which makes them unacceptable as models for studying human lipoprotein metabolism.

Response to High Fat Diet Challenge

Hamsters are also similar to humans in their response to increases in dietary cholesterol in that both will demonstrate an increase in serum LDL cholesterol (Jansen, 1989). When fed an appropriate diet, the hamster presents a more human-like lipoprotein profile than do many other rodent species [230, 238]. Hamsters fed a hyperlipidemic diet closely parallel the human type IV and type V hyperlipidmias in which serum triglyceride concentrations are increased [239].

In the absence of dietary cholesterol the VLDL + LDL/HDL ratio in the hamster is below 1.0 but this ratio increases after cholesterol feeding to 2.6 in the F_1B hamster strain [240]. Rats and mice, on the other hand, will respond to increases in dietary cholesterol by activating bile acid synthesis. Therefore, they primarily down-regulate hepatic synthesis and up-regulate hepatic bile acid production so that the plasma lipoprotein concentration remains relatively unchanged [236]. This response is distinctly different from humans, again making rats and mice poor models for dietary cholesterol challenge studies. Again, other animal models that behave similarly to humans in response to a dietary cholesterol challenge include guinea pigs, pigs, and several species of monkeys and baboon. An increase in cholesterol intake by 5 – 10 times their synthesis rate provides a rigorous cholesterol challenge to any of these animal models [236]. But again, the hamster is the more inexpensive model to feed and the easiest to handle making them most desirable.

On average, humans take in approximately 3 – 5 mg cholesterol/day/kg/ body weight which is equivalent to 50% or less of that synthesized. A comparable dose of dietary cholesterol in hamsters is achieved by adding only 0.1 – 0.3% cholesterol to the hamster's diet. If cholesterol is added at an amount higher than 0.3%, changes occur in liver biochemistry and plasma lipoprotein concentrations that do not have any relevance to the regulation of lipoprotein concentrations in humans [236]. In addition to cholesterol, high fat must be added to the diet in order for hamsters to develop combined hyperlipidemia. Cholesterol alone will not have this effect and neither does high fat alone [241]. The addition of cholesterol alone to the diet causes a dose-dependent increase in the level of cholesterol esters in the liver, a progressive suppression of LDL

receptor activity, and small increases in the LDL production rate. The addition of triacylglycerol containing saturated fatty acids to cholesterol containing diets lowers the concentration of hepatic cholesterol esters, further suppresses receptor activity, significantly increases the LDL production rate and as a consequence of these events, markedly raises the plasma LDL level [234, 235, 242, 243]. This is similar to the naturally occurring dietary situation in a large portion of the human population. The addition of saturated fat in the diet also reduces the uptake of cholesterol into the tissues through the LDL-R pathway [234, 242].

It takes about 4 -5 weeks for approximately 100 pools of LDL to be turned over in the hamster. This is when the hamster reaches a steady state in the distribution of unesterified and esterified cholesterol in the liver, the level of hepatic LDL receptor activity, the size of LDL production, and the concentration of LDL in the plasma. In humans this probably takes longer than 3 months to achieve [244].

Model for Human Atherosclerosis

While most hamster strains respond to high cholesterol diets with increased plasma LDL, one strain, the Syrian F_1B hamster is associated with an increased susceptibility to atherosclerosis due to a deficiency in LPL activity. The deficiency of LPL activity in the F_1B hamster causes an accumulation of chylomicrons and to a lesser extent VLDL [245]. The absence of functional LPL is one of the underlying causes of type 1 hyperlipoproteinemia in humans and it is possible that cholesterol may exert direct effects on LPL gene expression.

In addition, the hybrid F_1B hamster requires relatively low levels of dietary cholesterol to develop aortic atherosclerosis compared to other non-hybrid strains [246].

A diet containing 0.2% cholesterol and 10% coconut oil can cause fatty streak formation in the ascending aorta within 2 months [238]. Hypercholesterolemic diet-induced atherosclerosis in the hamster model is confined initially to a lesion-prone area along the inner curvature of the aortic arch [230, 238]. Prolonged consumption of an atherogenic diet results in the development of lesions throughout the aorta [245]. The lesions are similar to those found in humans [247, 248] characterized by the infiltration of monocytes, which become lipid-filled macrophage foam cells [246]. Within 12 months these early aortic lesions can develop into complex advanced plaques resembling human lesions, with a fibrous cap of SMCs, connective tissue matrix and a necrotic core containing cholesterol crystals [249]. Rats are resistant to atherosclerosis [250] and atherosclerotic lesions formed in any of the mouse models of atherosclerosis do not progress to the stage in which lesions promote the clinical manifestations observed in humans. Several species of rabbits develop atherosclerosis on high fat diets but the rabbit's cholesterol metabolism is different from that of humans and they develop a 'cholesterol storage disease' with lesions that are not the same as that of humans [251].

Model for Diabetic Accelerated Atherosclerosis

A major limiting factor in studying the mechanisms responsible for accelerated atherosclerosis in diabetes has been the lack of a suitable animal model. Diabetes appears spontaneously in some animal species, and several genetic models have been developed in rodents. However these animals are poor models of human diabetic atherosclerosis. The most successful approach to developing a diabetic atherosclerosis model has been to superimpose diabetes on a current atherosclerosis animal model such as the hyperlipidemic Syrian hamster. This has previously been accomplished by either

removing the insulin producing B-cell via pancreatectomy or by the injection of a B-cell toxin such as alloxan or streptozotocin (STZ).

STZ Induced Diabetes

STZ, a natural compound isolated from the gram positive bacterium *Streptomyces achromogene*, was discovered in 1960 as a new antibacterial antibiotic. It is an alkylating agent consisting of a methyl-nitrosurea side group attached to carbon-2 of D-glucose [252]. The glucose moiety allows for preferential uptake of STZ into B cells via the glucose transporter (GLUT)-2 [253, 254].

STZ exists as a mixture of alpha and beta anomers [255]. Upon dissolution of powdered STZ in an aqueous buffer, equilibrium between the two anomers is complete within 2 hours at room temperature with a nearly equimolar mixture of the anomeric forms [256]. STZ is unstable in unbuffered aqueous solutions, but stable for several days in a buffered aqueous solution with a pH of 4.0.

STZ has been used to induce diabetes mellitus in many different experimental animal models including the rat, mouse, guinea pig, monkey, pig and Chinese and Syrian hamsters [257-267] due to its selective pancreatic β-cell toxicity. However, most species have a high acute mortality to STZ injection, whereas Syrian hamsters show a high percentage of survival after STZ injection [268]. Han et al [269] determined that a single injection of 40 mg/kg of STZ was the appropriate dose to induce hyperglycemia in the APA strain of the Syrian hamster without nephrotoxicity although approximately 10% of the injected hamsters fail to develop hyperglycemia.

There is over 20 years of evidence supporting B cell death in STZ treated rodents [270-272]. STZ causes DNA strand breaks which then activate poly ADP-ribose

synthetase, an enzyme that polymerizes the ADP-ribose moiety of NAD to form poly ADP-ribose. This causes a lethal depletion of nicotinamide adenine dinucleotide (NAD) in the β-cells [273-278]. STZ has been shown to directly methylate DNA producing not only DNA strand breaks, but also alkali-labile sites, DNA adducts, chromosomal aberrations, micronuclei, sister chromatid exchanges, and cell death [279]. Free radicals may also be involved in the DNA and chromosome damage produced by STZ [280]. Gille et al. [281] demonstrated that STZ increases the generation of reactive oxygen species in murine islet cell cultures. STZ not only modulates energy metabolism by decreasing the amount of reducing equivalents such as NADH and NADPH but also by modulating oxygen metabolism in cells by reducing their antioxidant capacity and increasing mitochondrial ROS formation. Further aggravating the situation is the fact that intracellular metabolism of STZ also yields nitric oxide which precipitates additional DNA strand breaks [282]. The ROS induced damage of proteins, lipids and DNA, as well as the depletion of energy, all contributes to the death of the β-cells.

There are apparently essential characteristics that make the mature β-cell particularly susceptible to toxins such as STZ, one of which is the expression of GLUT - 2. However, the precursor population for both endocrine and exocrine tissue in the pancreas is not susceptible to damage by STZ [283]. This is most likely due to an insufficient expression of GLUT-2 on the immature cells.

As stated previously, Syrian hamsters of the APA strain are known to enter a marked and continuous diabetic state after a single injection of STZ without severe toxic effects. STZ-induced hamsters may be able to maintain this diabetic state due to the proliferation of immature precursor cells that are not affected by the STZ. This was

demonstrated by Wang et al. [284] in newborn rats injected with 100 ug/g of STZ. They found that while the STZ dramatically reduced the number of β-cells in islets it had little effect on the number of β-cells found in aggregates of less than six endocrine cells. Presumably, these small aggregates are neogenic and are derived from differentiation of a precursor cell population. The lack of an effect on these aggregates suggests that they may be a less differentiated, immature β-cell population not effected by STZ. Another possibility is that hyperglycemia may also play a role in stimulating β-cell replication after STZ administration. Glucose is a well-known stimulus for proliferation of pre-existing β-cells [285]. Movassat et. al. [286] demonstrated that hyperglycemia may also play a role in stimulating β-cell replication after STZ administration in newborn rats with partial pancreatectomy. However, whether the hyperglycemia was influencing preexisting mature β-cells or new β-cells arising from precursor cells was not determined. More recently, Finegood et. al. [283] demonstrated that prior STZ treatment did not inhibit pancreas regeneration after 90% pancreatectomy in rats. He concluded that there was no evidence that hyperglycemia enhanced the formation of new endocrine cells from ductal precursor cells but that the possibility that moderate hyperglycemia had a trophic influence on preexisting β-cells in the form of hypertrophy or replication could not be excluded. However he did find that severe hyperglycemia, or something associated with it, appeared to inhibit the development of the endocrine cell mass. Rosenberg [287] postulated that islet-cell regeneration in the diabetic hamster pancreas could be induced by a local growth factor(s). Whatever the reason, Takatori et al. [288], in a functional and histochemical analysis on pancreatic islets of APA hamsters with STZ-induced hyperglycemia and hyperlipidemia, found that these hamsters could remain in a diabetic

state because resident β-cells in the islets proliferated even after STZ injection and that most degenerated cells continued to maintain low levels of insulin secretion.

One problem with the use of STZ to induce the diabetic state is that the efficacy of STZ is highly variable between species, strain, age and laboratory. Even in the same species of animal receiving the same dose of STZ the results are widely variable [289]. This limits the predictability of the effects of STZ in inducing diabetes. Therefore other methods of inducing diabetes and insulin resistance are currently being investigated.

High Fat Diet Induced Insulin Resistance

Recently it has been determined that the high fat-fed Syrian Golden hamster without STZ injection, in addition to being a good model of human hyperlipidemia and exhibiting a significant weight gain, also exhibits hyperinsulinemia and hypertriglyceridemia which are characteristic of the profile often observed in obese insulin-resistant and/or type 2 diabetic patients [241]. The high fat diet load not only causes impairment of insulin secretion but also leads to insulin resistance making the high fat-fed hamster a model of nutritionally-induced insulin resistance [290]. The insulin resistance is accompanied by a marked increase in intestinal apoB48 particle production which is an integral feature of the insulin resistant state. Peripheral glucose disposal stimulated by insulin is inhibited by the elevated levels of plasma free fatty acids in man [291, 292]. The high-fat fed Syrian Golden hamster also has a significant reduction in whole body glucose disposal and a reduction in insulin suppression of hepatic glucose production [290].

High Fructose Diet Induced Insulin Resistance

Another model of nutritionally-induced insulin resistance is the high fructose-fed hamster. High fructose feeding alone for a 3-week period induces significant hypertriglyceridemia characterized by the overproduction of intestinal apoB48-containing lipoproteins in both the fasting and postprandial states [293]. Hyperinsulinemia and the development of whole body insulin resistance in the high fructose-fed Syrian golden hamster, similar to the high-fat fed model, is again due to the overproduction of apoB48 particles. However, in contrast to the high-fat fed model, the fructose fed hamster induces insulin resistance in the absence of significant weight gain [294]. The high fat high fructose fed hamster is therefore a good animal model for the study of the treatment of diabetic dyslipidemia [226]. This model also demonstrated insulin resistance as judged by elevated insulin levels during an oral glucose tolerance test (OGTT) indicating that it may be a useful model for the study of the interaction of diabetes and atherosclerosis. There may be less variability in response to high-fructose than there is to STZ. Additionally the use of fructose eliminates the hamster deaths related to the toxicity of STZ.

In conclusion, the hamster is a useful small animal model that simulates the interaction of diabetes and atherosclerosis because hyperglycemia and atherosclerosis can be easily induced and because the lipoprotein metabolism of hamsters has many attributes in common with humans. Additionally, hamsters develop atherosclerotic lesions and other diabetic complications similar to humans [295]. In the following study, combined diabetes and atherosclerosis was studied in STZ injected, high-fat fed Syrian F_1B hamsters.

CHAPTER V

MATERIALS AND METHODS

Animals, Diets, and Experimental Design

Sixty male golden F_1B Syrian hamsters (*Mesocricetus auratus*), 9 weeks old, weighing between 87 grams (g) and 106 g, were obtained from Biobreeders (Watertown, MA). Hamsters were housed individually in rodent cages under environmentally controlled conditions of 20 ± 1° C at 30% to 60% humidity on a 12-hour/12-hour light/dark cycle with *ad libitum* access to food and water. After one week of acclimation, animals were randomly divided into four experimental groups (Figure 1):

N – non-lipidemic/non-glycemic hamsters fed Purina 5001 standard rodent chow pellets containing 0.03% cholesterol and 4.5% fat (n=14);

L - lipidemic hamsters with lipidemia induced with 0.062 kgs purified cholesterol (0.25%) dissolved in 3 liters of hot coconut oil (10%) wt/wt (Research Diets, Inc., New Brunswick, NJ) and added to 22.7 kgs Purina 5001 rodent meal with the same formula as the standard rodent chow pellets (n =14).

G - glycemic hamsters fed standard chow with glycemia induced by one intraperitoneal injection of 40 mg/kg body weight streptozotocin (Stz) (Sigma Chemical, St. Louis, MO) freshly dissolved in 0.1M citrate buffer pH 4.5 (n =16); and

L+G - lipidemic/glycemic hamsters with lipidemia induced with the same diet as group L and glycemia induced with the same Stz injection as group G (n =16).

To prepare the high-fat diet, additional water was added to the final meal mix to achieve a moist consistency. This was compacted to a 2" depth in large Tupperware containers and frozen until just prior to feeding when it was defrosted and cut into squares.

All hamsters were weighed prior to the start of the study (time 0) and at 1, 2, 3, 6, and 8 weeks after the start of the study. All experimental procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of New Hampshire (IACUC approval #010702). Hamsters administered STZ had 5% glucose added to their water for two days in order to counteract the release of insulin from the dying beta cells and prevent diabetic shock. After Stz injection one hamster in the G group and four hamsters in the L+G group died.

After ten weeks, seven hamsters from each group were weighed, fasted overnight, and anesthetized with an intraperitoneal injection in the lower right abdominal quadrant of Ketamine-HCL:xylazine (20mg:8mg per 100 grams body weight) in a 1 cc tuberculin syringe (Becton Dickinson & Co., Rutherford, NJ). The hamsters were then euthanized by exsanguination. A small incision was made in the abdominal wall, through the peritoneum. The heart was exposed by making a longitudinal cut up to the sternum and through the ribs left of the sternum. Blood was withdrawn from the left ventricle with a 22 gauge 1 inch needle on a 3 cc syringe and was immediately aliquoted into one 200 ul Microvette 200 Z-gel tube for serum collection and two 1.3 mL Microtubes (Sarstedt, Newton, NC) containing 1.6 mg potassium-EDTA/ml of blood for plasma collection. Plasma samples were gently inverted several times and immediately spun at 1,500 x g for 15 minutes in an Eppendorf 5415 C microfuge (Brinkman Instruments, Inc., Westbury,

NY). Serum samples were allowed to clot for 20 to 30 minutes and then spun as above. After centrifugation, serum or plasma was removed and aliquots for lipids, glucose, and insulin analysis were placed on ice. All aliquots were frozen at - 80° C (Revco Scientific Inc., Asheville, NC) within 4 hours until analyzed.

To eliminate remaining blood cells and plasma proteins the vasculature of each hamster was immediately perfused with phosphate buffered saline (PBS) pH 7.4 by the insertion of a 21 gauge 1 inch butterfly needle into the left ventricle and a cut in the right atrium to allow an outlet for the solution. The saline was perfused for 5 minutes at the physiologic pressure of 100 to 120 mm Hg. Following the saline perfusion, four hamsters from each group were put in a plastic bag on ice. The remaining three hamsters from each group were perfused with 10% neutral buffered formalin (NBF) for 10 minutes under the same conditions as used for the saline. After 10 minutes of fixation *in situ*, the hamsters were put in a plastic bag on ice. The four hamsters from each group that were perfused with PBS only were dissected to remove the heart and aorta and a section of liver and kidney which were then placed in a vial and flushed with nitrogen gas before being snap frozen in liquid nitrogen and stored at minus 80° C. The three hamsters that were additionally perfused with NBF were also dissected to remove the heart, and aorta, and sections of liver, kidney and pancreas. The heart and aorta were pinned out at *in situ* length and all tissue was immersed in NBF overnight. After overnight fixation the aortic arch was cut into three sections of approximately 3 mm in length, proximal, medial and distal to the heart. Each tissue section was placed between two foam pads in a plastic embedding cassette and dehydrated and infiltrated overnight in the Citadel 2000 (Shandon) carousel style automatic tissue processor. Heat and vacuum were not utilized

in the tissue processing. The clearing agent used was Histoclear II (National Diagnostics, Atlanta, GA) and the paraffin used for impregnations was Paraplast Plus (Fisher Scientific). The embedded tissue cassettes were kept refrigerated for future sectioning. This procedure was repeated at week 20 on the remaining seven hamsters from groups N and L, five hamsters from group L+G, and eight hamsters from group G. In addition, plasma samples were taken from five hamsters in the N, G and L groups and four hamsters in the L+G group at 20 weeks for lipid hydroperoxide analysis. Lipid hydroperoxides were extracted that same day and the extracted samples frozen at - 80° C.

At two weeks and ten weeks after STZ injection blood glucose levels were evaluated using the FastTake glucometer. Hamsters with fasting blood glucose levels greater than 200 mg/dL at two weeks after injection (WAI) and greater than 290 mg/dL at 10 WAI were considered hyperglycemic. Two hundred and ninety mg/dL of glucose represented a 62% increase in fasting glucose values over the non-treated hamsters average glucose values at week 10. In humans, only a 26% increase in fasting glucose values on more than one occasion is indicative of diabetes, therefore, the criteria used for the hamsters in this study was fairly strict. Since the hamsters glucose values varied by ±30 mg/dL, this strict criteria assured that the glucose values between the non-treated hamsters and the glycemic hamsters were significantly different at the $p<0.001$ level. Four STZ injected hamsters did not meet the criteria and were eliminated from the study.

Lipid Analysis

Plasma aliquots for lipid analysis were thawed at room temperature (RT), diluted 1:2 with physiological saline solution (The Butler Company, Columbus, OH), and briefly

vortexed. Total cholesterol (TC) levels were determined by a cholesterol oxidase enzymatic method (INFINITY ™ Cholesterol Reagent procedure No. 402, Sigma Diagnostics, St. Louis, MO). Samples with TC beyond the assay range of linearity (750 mg/dL) were further diluted with physiological saline to a final dilution of 1:8 and re-assayed. Results were then multiplied by the dilution factors. High density lipoprotein cholesterol (HDL-C) levels were determined by precipitation of both low density lipoprotein cholesterol (LDL-C) and very low density lipoprotein cholesterol (VLDL-C) with phosphotungstic acid in conjunction with magnesium chloride (HDL Cholesterol Reagent (PTA/$MgCl_2$) procedure No. 352-4, Sigma Diagnostics, St. Louis, MO). The cholesterol concentration in the remaining HDL fraction was then assayed by the same enzymatic method used to determine TC. The combined cholesterol in the VLDL and LDL fractions (non-HDL-C) was calculated as the difference between TC and HDL. LDL-C could not be calculated by the Friedwald method as triglycerides in the L and L+G groups often exceeded 400 mg/dL. This increased level of triglycerides interferes with the accuracy of the Friedwald calculation. Triglyceride levels were determined by an enzymatic method (INFINITY ™ Triglycerides Reagent procedure No. 344, Sigma Diagnostics, St. Louis, MO). Samples with triglycerides beyond the assay range of linearity (800 mg/dL) were further diluted with physiological saline to a final dilution of 1:10 and re-assayed. Results were multiplied by the dilution factors and quantified spectrophotometrically (Spectronic Genesys 5, Milton Roy Company, Rochester, NY).

Glucose Analysis

Serum aliquots for glucose analysis were thawed at RT and briefly vortexed. Glucose levels were determined by a hexokinase enzymatic method (Glucose (Trinder)

Reagent procedure No. 315, Sigma Diagnostics, St. Louis, MO). Since turbid samples may give falsely high values a sample blank was used with all turbid samples according to the recommended sample blank procedure. The sample blank was prepared by adding the sample to physiologic saline and the absorbance of the sample blank was subtracted from the absorbance of the sample assayed with the glucose reagent in order to obtain the actual glucose concentration. Results were quantified spectrophotometrically (Spectronic Genesys 5, Milton Roy Company, Rochester, NY).

Lipid Hydroperoxide (LPO) Analysis

Extracted plasma samples from week 20 for LPO analysis were thawed on ice. Quantification of lipid peroxidation was determined using a Lipid Hydroperoxide Assay Kit (Cayman Chemical Co., Ann Arbor, MI) that measures the hydroperoxides directly by utilizing redox reactions with ferrous ions. Hydroperoxides are highly unstable and react readily with ferrous ions to produce ferric ions. The resulting ferric ions were detected using thiocyanate ion as the chromogen. Results were quantified on a spectrophotometric plate reader (EL_x800, Bio-Tek Instruments, Inc., Winooski, VT) at 490 nm.

Insulin Analysis

Plasma samples for insulin analysis were thawed at RT. Quantification of insulin was determined using a rat insulin radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, MI) utilizing insulin labeled with ^{125} Iodine and a rat insulin antiserum raised in guinea pigs to determine the level of insulin by a double antibody/PEG technique. According to the manufacturer this antibody has been shown to have 100% specificity for

hamster insulin. After performing the assay, remaining radioactive counts per minute were determined (LKB WALLAC 1282 Compugamma Universal Gamma Counter, Gaithersburg, MD) and a standard curve was plotted using nonlinear regression. Insulin levels in ng/mL were determined.

Statistical Analysis

Student's *t*-test with a two-tailed measurement of P values was used to compare each group's lipid, glucose, LPO, and insulin values between the 10 week timepoint and the 20 week timepoint. One-way factorial analysis of variance (ANOVA) followed by Tukey's Multiple Comparison post test was used to compare each group's lipid, glucose, LPO, and insulin values within each timepoint. All analysis was done using the V2.0b GraphPad Prism (GraphPad Software, Inc) statistical program. Data was expressed as mean $\pm$ SD. Values of $P < 0.05$ were considered statistically significant. If the F test was significant, differences between subsets were performed on logarithmically transformed data. For correlation data, Spearman correlation coefficients were calculated on logarithmically transformed data.

Immunohistochemical Analysis

Immunohistochemical (IHC) analysis was performed on formalin fixed, paraffin embedded sections cut at 5μm and mounted on Superfrost Plus™ slides. Subsequently the paraffin was removed by placing slides in two 10 minute baths of toluene followed by rehydration of the tissue sections via 6 minute baths in 100% EtOH, 95% EtOH, ddH_20 and final submersion in PBS.

Slides containing sections of pancreas from hamster groups N, L, and G were first incubated for 5 minutes in 3% H_2O_2 in water to quench endogenous peroxidase activity and then rinsed in PBS for 5 minutes. All incubations took place in a humidified chamber. Sections were then blocked with dilute goat serum contained in the VECTASTAIN® Elite® ABC Peroxidase Kit (Vector Laboratories, Inc., Burlingame, CA) and incubated at room temperature for 20 minutes. Excess serum was blotted from the sections. The sections were then incubated overnight at 4 ° C with a rabbit IgG polyclonal primary antibody to porcine insulin (ICN Pharmaceuticals, Inc., Aurora, Ohio) diluted with PBS containing 0.1% immunohistochemical grade bovine serum albumin to prevent adsorption of the antibody to the plastic microfuge tube in which the final dilution was made. This antibody is known to react with cytoplasmic and extracellular insulin in humans, pigs and rats. After rinsing in PBS for 5 minutes a biotinylated goat anti-rabbit IgG secondary antibody was added. This introduced biotin into the section at the location of the primary insulin antibody. After rinsing in PBS for 5 minutes an avidin:biotinylated peroxidase enzyme complex (ABC) was added. This bound to the biotinylated secondary antibody. Sections were again rinsed in PBS for 5 minutes and then Vector® VIP peroxidase substrate solution was added until the desired purple stain intensity developed. The section were rinsed in tap water and counterstained with Vector® Blue, cleared and mounted.

IHC analysis was similarly performed on slides containing sections of the aortic arch from hamster groups N, L, G, and L+G using an affinity-purified goat polyclonal antibody, ox-LDL receptor-1 (also designated LOX-1) diluted 1:10 with PBS with 1% bovine serum albumin. This antibody was raised against a peptide within an internal

region of ox-LDL receptor-1 of mouse origin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and previously had been demonstrated to react with mouse and rat tissue. A Vectastain® ABC Alkaline Phosphatase enzyme kit was used containing a biotinylated anti-goat IgG secondary antibody make in rabbit. The substrate used was Fast Red (Signet Labs, Inc., Dedham, MA) and the counterstain used was Hematoxylin QS (Vector Laboratories, Inc., Burlingame, CA).

Following the same protocol IHC analysis was performed on hamster aortic sections using a rabbit anti-MDA antibody (Alpha Diagnostic International, San Antonio, TX) in which the MDA was coupled with keyhole limpet hemacyanin (KLH) prior to injection into rabbits. This antibody was also known to react with mouse and rat tissue.

Serial sections were used for all IHC protocols and run simultaneously applying 1% bovine serum albumin in PBS in place of the primary antibody. Peptide neutralization was also performed using the peptide that the LOX-1 antibody was raised against (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The peptide was reconstituted in PBS to a concentration of between 0.2 – 1 mg/mL. Antibody at the same 1:10 dilution as used in the IHC protocol was combined with a ten-fold excess of peptide and incubated for 2 hours at RT. The antibody-peptide complexes were centrifuged at full speed in a microfuge for 15 minutes before use. During the IHC protocol the supernatant antibody-peptide complexes were applied to duplicate sections in place of the LOX-1 antibody.

Photodocumentation

Representative sections from each group were examined using the 20X objective on an Olympus BHS-RFC microscope (Olympus America, Inc., Lake Success, NY).

Images were captured using a QImaging CCD MicroPublisher digital camera and QCapture software (QImaging, BC, Canada).

CHAPTER VI

RESULTS

Body Weights

The average initial hamster weight of for all hamsters was 95.79 grams. The average weight increased steadily through 8 weeks of treatment to 106.5 grams. Hamsters sacrificed at week 10 had an average weight of 103.9 grams and hamsters terminated at week 20 had an average weight of 106.7 grams. There was no significant difference in the average weight of the 4 groups at the start of the study. There were also no significant differences in the initial weights within each group between hamsters at week 10 and hamsters at week 20.

Group Differences. At the 10 week timepoint the hyperlipidemic/normoglycemic (L) hamsters had significantly higher ($p<0.05$) body weights than the normolipidemic/normoglycemic (N) hamsters. While the mean weight of each group at the 10 week timepoint had increased over the mean initial body weight only the hyperlipidemic/normoglycemic (L) hamsters significantly increased their weight ($p<0.001$). There were no other significant differences between the groups at the 10 week time point. At the 20 week timepoint there were no significant differences in body weights between groups.

Timepoint Differences. While the average body weight of each group at 20 weeks had increased over the average initial weight only the normolipidemic/normoglycemic (N) hamsters body weight increased significantly ($p < 0.01$). Between the 10 week timepoint and the 20 week timepoint the average weight of the normolipidemic/normoglycemic (N), normolipidemic/glycemic (G) and hyperlipidemic/hyperglycemic (L+G) groups was higher at week 20 while the average weight of the hyperlipidemic/normoglycemic (L)

group was lower at week 20. However, there were no significant differences within each group at the week 10 timepoint versus the week 20 timepoint (Table 1).

Lipid Analysis

Group Differences. Comparisons between the four groups, at the 10 week timepoint and the 20 week timepoint produced the following results:

Total Cholesterol and non-HDL-C. The L group had significantly higher TC and non-HDL-C than the N or G groups at both week 10 ($p < 0.001$) and week 20 ($p < 0.001$). The L+G group had significantly higher TC and non-HDL-C than any other group at both week 10 ($p < 0.001$) and week 20 ($p < 0.001$). There was no significant difference in TC and non-HDL-C between the N group and the G group at either the 10 week or 20 week timepoint (Tables 2, 3, & 4, Graphs 1 & 2).

HDL-C. The L group had significantly higher HDL-C than the N or G group at both week 10 ($p < 0.05$) and week 20 ($p< 0.001$). The L+G group had significantly higher HDL-C than any other group at both weeks 10 ($p < 0.001$) and 20 ($p < 0.001$). At week 10 there was no significant difference in HDL-C between the N and G groups but at week 20 HDL-C in the G group was decreased significantly compared to the N group ($p < 0.01$) (Tables 2, 3, & 4, Graph 3).

Triglycerides (TG). The L and G groups had significantly higher triglycerides than the N groups at both week 10 ($p < 0.001$) and 20 ($p < 0.05$). The L+G group had significantly higher triglycerides than any other group at both week 10 ($p < 0.001$) and 20 ($p < 0.001$). There was no significant difference in the triglycerides between the L and G group at these timepoints (Tables 2, 3, & 4, Graph 4).

TC/HDL-C. The L group had a significantly higher TC/HDL-C ratio value than the N group at both weeks 10 ($p < 0.001$) and 20 ($p < 0.01$) and a significantly higher ratio value than the G group at weeks 10 ($p < 0.001$) and 20 ($p < 0.05$). The L+G group had a significantly higher TC-HDL-C ratio than the N and G groups at week 10 ($p < 0.001$) and

week 20 ($p < 0.001$) and significantly higher than the L group at week 10 ($p < 0.001$) and week 20 ($p < 0.05$). There was no significant difference in the TC-HDL-C ratio between the N and G groups at either time (Tables 2, 3, & 4, Graph 5).

TG/HDL-C. At the 10 week timepoint the L group had almost a 3-fold increase in the average TG/HDL-C ratio over the N group. The G group had almost a 5-fold increase in the average TG/HDL-C ratio over the N group at the week 10 timepoint. Also at the week 10 timepoint the L+G group had a 34.5-fold increase in the average TG/HDL-C ratio over the N group, which was also a 7-fold increase over the G group and almost a 12-fold increase over the L group. At the 20 week timepoint the L group had a 1.7-fold increase in the average TG/HDL-C ratio over the N group. The G group had a 3-fold increase in the average TG/HDL-C ratio over the N group at the 20 week timepoint. Also at the 20 week timepoint the L+G group had a 10-fold increase in the average TG/HDL-C ratio over the N group, a 3.5-fold increase over the G group and a 5.8-fold increase over the L group (Table 2, Graph 6).

Timepoint Differences. Comparisons within the four groups, between the two time points showed that the only significant differences within the groups when comparing the lipid levels of hamsters at week 10 versus those at week 20 occurred in the N group. At the 20 week timepoint the TC, non-HDL-C, and TC/HDL-C in the N group were significantly higher ($p < 0.05$) than at the 10 week timepoint and the triglycerides in the N group were significantly higher ($p < 0.01$) at the 20 week timepoint than at the 10 week tiempoint (Table 2, Graphs 1, 2, 3, 4, 5, & 6).

Glucose Analyses

Group Differences. Comparisons between the four groups at the two time points showed that the L+G and G groups had significantly higher glucose levels than the N group at both weeks 10 ($p < 0.001$) and 20 ($p < 0.001$). The L+G and G groups also had significantly higher glucose levels than the L group at weeks 10 ($p < 0.01$) and 20 ($p <$

0.001 and $p < 0.05$ respectively). At week 10 the L group had significantly higher ($p < 0.01$) glucose levels than the N group but at week 20 there was no significant difference in glucose between the N and L groups. There was no significant difference in the glucose levels between the G and L+G group at either time (Tables 2, 3, & 4, Graph 7).

Timepoint Differences. There were no significant differences in glucose values of the N group between weeks 10 and 20, the G group between weeks 10 and 20, the L group between weeks 10 and 20, or the L+G group between weeks 10 and 20 (Table 2, Graph 7).

Insulin Analyses

At week 10 the L+G and the G groups had significantly increased insulin levels over the N group ($p<0.001$). The L group also had significantly increased insulin over the N group ($p<0.05$). The L+G group had significantly increased insulin over the L and the G groups ($p<0.001$). There was no significant difference in insulin between the G and L groups.

At week 20 the L+G group had significantly increased insulin levels over the N group ($p<0.001$). Although the L and G groups still had glucose values higher than the N group at week 20 it was no longer significant due to an increase in the insulin levels in the N group from week 10 to week 20. The L+G group still had significantly increased insulin over the L and G groups ($p<0.001$). The G group at this time had significantly elevated insulin over the L group ($p<0.05$) at the 20 week timepoint due to an increase in insulin levels in the G group and a decrease in insulin levels in the L group from the 10 week values (Tables 2, 3, & 4, Graph 8).

Lipid Hydroperoxide (LPO) Analysis

At the 20 week timepoint mean LPO values (uM) for each group were as follows: 5.13 in the N group, 2.81 in the G group, 5.24 in the L group and 7.80 in the L+G group. The L+G group had significantly higher ($p < 0.01$) LPO values than the G group. There

were no other significant differences between groups (Graph 1). Correlation between the log of total cholesterol and the log of lipid hydroperoxides in N, G, L and L +G hamsters at 20 weeks had an r value of 0.5033 and a p value of 0.0237 (Graphs 9 & 10).

Pancreatic Islet Histopathology

At the 10 week timepoint the islets in the pancreata of the L group are slightly smaller and there were fewer islets than in the pancreata of the N group. In addition the beta cells in the L group appeared less dense and vacuolated when compared to the beta cells in the pancreas of the N hamsters. The islets in the pancreata of the G group at the 10 week timepoint were even smaller and fewer and the beta cells even more vacuolated than those of the L group (Figures 2 & 3).

Pancreatic Islet Immunohistochemistry

In the N group, cells immunoreactive for insulin designated by the dark purple stain are distributed throughout the islets. Insulin immuno-reactivity is greatly decreased in the islets of the age matched L group. Even though the islets in the G group were smaller and more vacuolated than the N and L group the immuno-reactivity appears to be similar to that of the L group (Figures 2 & 3).

Aortic Arch Lesion Development

At 20 weeks there was no visible atherosclerotic lesion development in the N or G groups. The L group had early stage, fatty streak lesions with foam cell formation. The most striking lesion development occurred in the L+G group where intermediate stage, fibro-muscular lesions included features of advanced stage, complicated lesions. These lesions had visible SMC proliferation characteristic of the fibro-muscular stage of lesion development. The core of these lesions was missing, having fallen out during sectioning.

This indicates that the core most likely consisted of dead cells and gruel characteristic of more advanced lesions (Figure 4).

Immunohistochemistry for MDA and LOX-1

MDA. There was very faint immuno-reactivity for MDA in the N group with a few focal areas in the SMCs and possibly some EC immuno-reactivity. There was moderately intense immuno-reactivity for MDA in the SMCs of the G group and visible EC immuno-reactivity. The foam cells in the L group were highly immuno-reactive but the SMCs had similar reactivity to the N group. The L+G group intense immuno-reactivity within the core of the lesion, and in ECs and SMCs (Figure 5).

LOX-1. The N group had immuno-reactivity for LOX-1 within the SMCs with a few focal areas and the appearance of some EC immuno-reactivity. There was moderately intense immuno-reactivty for LOX-1 in the SMCs of the G group and visible EC immuno-reactivity. However, not only were the foam cells in the L group moderately to intensely immuno-reactive but the SMCs were also highly immuno-reactive. The L+G group was intensely immuno-reactive within the core of the lesion and also throughout the SMCs (Figure 6).

CHAPTER VI

DISCUSSION

Type I and Type II diabetics are prone to the same risk factors for developing atherosclerosis as non-diabetics, however their risk is increased by a factor of 2 to 5. In order to study the mechanism(s) responsible for this accelerated atherosclerosis in diabetics development of a suitable animal model of diabetic atherosclerosis is necessary. While genetic models of diabetes have been developed in rodents, and other animal species may develop diabetes spontaneously, they are not good models of diabetic atherosclerosis since they either do not develop lesions similar to humans or they have lipid and lipoprotein metabolism and profiles that are different from humans. One method that has been determined to be useful is to take an animal that is a good model for human atherosclerosis and superimpose diabetes. Initially pancreatectomy was utilized to simulate diabetes, however, without the administration of insulin these animals would not live long enough to develop atherosclerosis. A second method, the administration of diabetogenic agents that cause B-cell ablation such as STZ, has been used in animal models. A diabetic state has previously been induced by STZ in the APA strain of the Syrian hamster extensively used in Japan [296].

Objective 1

The first objective of this study was to determine whether one intraperitoneal injection of 40 mg/kg body weight of STZ could induce a diabetic-like hyperglycemic

state in the Syrian F_1B hamster, an atherosclerotic hamster model used in the United States, and whether the diabetic-like hyperglycemic state was similar to human Type I or Type II diabetes.

Hyperglycemia is the primary clinical manifestation of both Type I and Type II diabetes. The comparison of hamster fasting glucose values between the STZ injected hamster groups (G and L+G) and the non-STZ injected hamster groups (N and L) indicated that one intraperitoneal injection of 40 mg/kg body weight of STZ was successful in creating a hyperglycemic state in the Syrian F_1B hamster. The G and L+G groups injected with STZ had significantly higher fasting plasma glucose levels than the N and L groups, at both weeks 10 and 20. After STZ injection, the fasting plasma glucose level of the STZ-treated groups (G and L+G) was 1.75 – 2.75 times higher than those of the non-treated hamsters. In similar studies with APA hamsters [288] non-fasting serum glucose levels of STZ treated hamsters were 2 – 3 times as high as those of control animals. These results indicate that a single injection of STZ caused hyperglycemia similar to that found in human diabetes in the absence of insulin administration.

Of interest was that the L group also had significantly elevated glucose levels over the N group at week 10 but not at week 20. This lack of significant difference at week 20 was due to an elevation in the fasting glucose levels of the N group at 20 week and not to a decrease in glucose values in the L group. Elevated glucose in hyperlipidemic hamsters was also previously demonstrated in studies of the APA hamster strain [280].

In addition to hyperglycemia, another characteristic of human diabetes is dyslipidemia characterized by hypertriglyceridemia, decreased plasma HDL, and increased small, dense LDL particles, the so-called "atherogenic lipid triad" [297-299]. However human diabetics may not necessarily have an increase in total cholesterol or LDL-C concentrations. In this study there was no significant difference in total cholesterol between the N and G groups. This differs from what was seen previously by Simionescu et al. [280] in the APA strain of the Syrian hamster where higher total cholesterol in the STZ treated hamsters was observed. There was also no significant difference in non-HDL-C cholesterol between the N and G groups. This lack of significant increase in both total cholesterol and non-HDL-C in the F_1B STZ treated hamsters is similar to what is seen in human diabetes.

The G group also had approximately a 4.6-fold increase in triglycerides over the N group at the 10 week timepoint but only a 2.1-fold increase at the 20 week timepoint. This was due more to an increase in triglycerides in the N group as they aged as opposed to a decrease in the triglycerides in the G group. Ebara et al. [300] also observed increases in the triglycerides of STZ treated APA hamsters by 5.6-7.8-fold over normal hamsters just 10 days after STZ injection. The striking elevation in plasma triglycerides seen in the STZ treated F_1B hamster is similar to what is observed in human diabetes.

At 10 weeks there was also no significant difference between the HDL-C levels of the N and G groups. However by 20 weeks the G group had significantly decreased HDL-C from the N group. This was also previously seen in STZ treated APA hamsters [296]. Again, this decrease in HDL-C is similar to what has been reported in human diabetes.

While there may be no increase in absolute non-HDL-C fraction which would contain LDL in the G group, increases noted in the TG/HDL ratio over the N group indicates the presence of larger amounts of small, dense LDL in the G group. At week 10 the G group had approximately a 5-fold increase in this ratio over the N group and at 20 weeks approximately a 3-fold increase over the N group. This increase in small, dense LDL is similar to what is seen in human diabetics. In this study, the STZ treated Syrian F_1B hamster exhibited not only the hyperglycemia similar to that seen in non-treated human diabetics but also a similar lipid profile to that prevalent in human diabetes, making it a relevant model of human diabetes.

Determining whether STZ injection resulted in a model with characteristics similar to human Type I or Type II diabetes was more complicated. In humans, Type I diabetics have an absence of insulin production and Type II diabetics are characterized by an initial increase in insulin production in an attempt to compensate for a decreased peripheral sensitivity to the insulin. This initial increase in insulin is followed by a slow decline in insulin levels with the Type II diabetic eventually becoming hypoinsulinemic and hyperglycemic. It was anticipated in this study that STZ injection would destroy the beta cells. If all the beta cells were destroyed then no insulin would be produced. Under these circumstances the hamsters would not have survived without the administration of exogenous insulin. One of the reasons it had previously been determined that hamsters could survive for a relatively long time after STZ treatment without the administration of exogenous insulin was because of their continued secretion of insulin at a high enough level to allow enough glucose to enter into the cells of the body [301]. In this study the STZ treated hamsters not only continued to secrete insulin but had statistically significant

increased insulin levels over the N group at both weeks 10 and 20 and over the L group by week 20. The plasma insulin levels indicated a hypersecretory state similar to that which occurs initially in human Type II diabetes.

In order to determine whether the beta cells of the STZ injected hamsters were still producing insulin, sections of pancreata were reacted with an anti-insulin antibody and visualized with chromagen. While the beta cells themselves appeared vacuolated and the size of the islets smaller than the non-treated hamsters the immunoreactivity for insulin was evident in islet remnants. The lack of dependence on insulin, and its apparent continued secretion from the beta cells in the STZ treated Syrian F_1B hamsters appeared to be more reflective of type II diabetes rather than type I diabetes. It has been suggested by others that the L+G hamster manifests the conditions of both insulin resistance and insulin impairment [302] as seen in human type II diabetes.

Objective 2

The second objective of this study was to compare the development of atherosclerotic lesions between normal non-treated (N), hyperglycemic (G), hyperlipidemic (L) and combined hyperlipidemic/hyperglycemic (L+G) hamsters.

During the course of this study neither the N (Figure 1A) nor the G group (Figure 1B) developed atherosclerotic lesions. The L group (Figure 1C) developed early fatty streak lesions characterized by foam cell accumulation. The most striking findings were the extremely more advanced fibro-muscular lesions observed in the L+G group (Figure 1D). These fully developed intermediate stage lesions included SMC proliferation characteristic of the fibro-muscular stage of lesion development. In addition, the SMCs migrated to form a cap over a large vacant core. While this core was pulled out during

sectioning it most likely consisted of dead cells and extracellular matrix. This core is characteristic of more advanced lesions.

Objective 3

The third objective of this study was to determine whether any of the following factors influenced atherosclerotic lesion development in any of the four groups: glucose, triglycerides, total cholesterol, Non-HDL-C, HDL-C, TC/HDL-C ratio, TG/HDL-C ratio as an indicator of small dense LDL production, lipid hydroperoxides in the plasma, and MDA and LOX-1 in the vascular wall of the hamster aortic arch.

The L+G group had significantly greater lesion development than all other groups. The L+G group had fully developed fibro-fatty lesions by 20 weeks while the G group had no lesion development. In relation to the L+G group the L group had less advanced fatty or early streak lesions. In order to focus on what might have influenced this apparent accelerated atherosclerotic lesion development in the L+G group, plasma glucose, insulin, lipids, and lipid hydroperoxides were measured and staining for MDA and LOX-1 in the aortic arch was performed. It is important to note that at 20 weeks there were no significant differences in the weights between groups (Table 1). This eliminated weight as a confounding factor in any of the studies findings.

Glucose. The view that many of the pathologic effects of diabetes are from the condition of hyperglycemia itself is widely held. However, it should be noted that increased lesion development is already noted upon diagnosis of diabetes indicating that these lesions are most likely developing during the euglycemic pre-diabetic state.

Hyperglycemia, unique to patients with diabetes and impaired glucose tolerance, has been examined as a potential atherogenic factor. Hyperglycemia has been shown to

increase glycation of proteins and nucleic acids and favor oxidative reactions in the microenvironment of the artery wall [303] leading to the speculation that the origin of diabetic vascular complications is the formation of AGEs and oxidative stress. Actually it is very difficult to separate these two mechanisms. Because the production of many of the AGEs involves oxidation and glycation the term glycoxidation is often used when referring to this process in diabetics. AGES are therefore glycated proteins, lipids, or nucleic acids that have been oxidatively modified or glycoxidized due to hyperglycemia and the increased oxidative stress caused by the hyperglycemia [304]. Proteins may be further damaged by hydroxyl radicals released during glucose auto-oxidation. High glucose levels have also been shown to stimulate ROS through the PKC-dependent activation of NADPH oxidase and to stimulate mitochondrial superoxide production further increasing the oxidative stress in diabetics [305, 306]. Diabetes may increase mitochondrial generation of ROS through some diabetes associated mitochondrial dysfunction. In addition elevated glucose has been shown to increase the expression and activity of 12-lipoxygenase in SMCs.

AGEs form in LDL primarily by glycosylation of its phospholipids [307] but there is also the potential of glycosylation of the apoprotein B-100 molecule [308]. Increased glycated LDL has been demonstrated in the circulation and the vascular wall of diabetics and has been shown to be very sensitive to oxidation [309, 310]. It has been suggested that glycoxidized LDL is more rapidly taken up by macrophages than just oxidized LDL [311]. This modification of lipoproteins could therefore be one of the mechanisms for the early development of atherosclerosis in diabetics.

RAGE, the receptor for AGE, is also increased in diabetes. The binding of AGE to RAGE increases oxidative stress and activation of the inflammatory response in the vasculature. AGE-RAGE interaction causes activation of NF-*k*B. This system is subject to a positive feedback loop in that the binding of RAGE by AGEs leads to the upregulation of more RAGE through this NF-*k*B dependent mechanism. When endothelial cell RAGE binds AGE, NF-*k*B translocation to the nucleus activates the gene expression of VCAM-1 [312, 313]. The increased expression of adhesion molecules in diabetics may further contribute to accelerated atherogenesis [314, 315].

In this study, the fasting plasma glucose values between the G group and the L+G group were not statistically different indicating that any differences in lesion development between these two groups was not due to a direct effect of glucose alone. Elevated glucose in hyperlipidemia has also been previously demonstrated in studies of the APA hamster strain [280]. It appears that glucose alone may not be a factor in the accelerated development of atherosclerosis in the L+G group.

Total Cholesterol, HDL-C, Non-HDL-C, and TC/HDL-C ratio. There was no statistical difference between the G group and the N group with regards to TC, HDL-C, non-HDL-C and TC/HDL-C ratio the week 10 timepoint. A higher TC/HDL ratio is generally indicative of a more proatherogenic lipid profile. At week 20 the only significant change was that the glycemic hamsters now had significantly lower levels of HDL-C than the non-treated hamsters. HDL-C is responsible for the removal of cholesterol from the tissues. Type II diabetics often have low HDL-C levels which may be partly responsible for the increased risk of atherosclerosis in diabetics [316]. However

the decrease in HDL-C levels was not enough to cause a significant change in the TC/HDL-C ratio.

In contrast to the G group, the L+G group had significantly higher total cholesterol, HDL-C, Non-HDL-C and TC/HDL-C ratios compared to the other three groups. The G group had no visible lesion development whereas the L+G group had more advance atherosclerosis compared to the L group. These results indicated that increased plasma lipoprotein levels may be responsible for the accelerated atherosclerosis in the L+G group and that the TC/HDL-C ratio may indeed be an accurate indicator of the development of atherosclerosis in the Syrian F_1B hamsters.

Triglycerides. The hypertriglyceridemia seen in the G and L+G groups could be due to either the increased production of VLDL and chylomicrons, their decreased clearance, or both. A previous study determined that the hypertriglyceridemia seen in diabetic hamsters was not due to a difference in the amount of food consumed and that the hepatic output of triglycerides was actually decreased in diabetic hamsters. The study also determined that the increase in triglycerides was associated with a catabolic defect of VLDL triglyceride [300] not an increase in the production of VLDL. In addition to a decrease in the catabolism of VLDL triglycerides, chylomicron accumulation in the plasma due to impaired clearance could also be a contributing factor to the increased concentration of triglyceride-rich lipoproteins in the diabetic hamster.

In a previous study, diabetic hamsters demonstrated reduced lipoprotein lipase (LPL) activity by about one half and this deficiency in LPL activity was associated with an increase in susceptibility to atherosclerosis in the F_1B hamster [245]. LPL is a glycoprotein synthesized and secreted by macrophages and other cells [317]. This

enzyme adheres to the luminal side of vascular endothelial cells and hydrolyzes triglycerides in chylomicrons and VLDL thereby converting these to denser lipoproteins such as chylomicron remnants, intermediate density lipoprotein (IDL) and LDL. LPLs effect on the development of atherosclerosis is uncertain. Recent studies have revealed that LPL on macrophages and in the plasma may have different roles with different effects on the development of atherosclerosis [84]. Highly oxidized but not native or mildly oxidized LDL downregulates LPL production by macrophages [318]. Several studies have provided evidence that LPL secreted by macrophages promotes foam cell formation and accelerates atherosclerosis [319, 320].

Increased fasting triglyceride levels are characteristic of human diabetes. There is mounting evidence that elevated triglycerides in diabetics constitute a risk factor for atherosclerosis. Several studies have demonstrated that high levels of triglyceride rich lipoproteins and delayed remnant removal may increase monocyte adherence to endothelial cells inducing atherogenesis [321, 322]. In patients with hypertriglyceridemia, serum levels of soluble E-selectin, ICAM-1 and VCAM-1 were shown to be elevated independently of other risk factors [323, 324]. There is also some evidence that triglyceride-rich lipoproteins readily cross the endothelial cell barrier [134, 325].

All three treated groups of hamsters (G, L, and L+G) had significantly increased triglycerides over the non treated hamsters. The L and G groups had approximately a 4 - 5-fold increase at the 10 week timepoint but only a 2 - 3-fold increase at the 20 week timepoint. This was due to an increase in triglycerides in the N group at 20 weeks as opposed to a decrease in the triglycerides in the G and L groups. Ebara et al. [300] also

observed increases in the triglycerides of STZ treated hamsters by 5.6 – 7.8-fold over normal hamsters just 10 days after STZ injection.

There was an even greater increase in the triglycerides of the L+G group. Their triglycerides were increased 150-fold over the N group at 10 weeks but were increased only 66.5-fold over the N group at 20 weeks. The triglycerides in the L+G group were also increased 34-fold over the L and the G groups at 10 weeks and approximately 28-fold at 20 weeks indicating that the combination of hyperglycemia and hyperlipidemia had a synergistic effect on the level of triglycerides.

It has been observed that elevated plasma glucose often produces similar triglyceride levels to high saturated fat diets in humans but with substantially lower serum LDL cholesterol concentrations [326]. Indeed in this study there was no significant difference between the triglycerides in the L and the G groups. Since the L group developed foam cell lesions but the G group had no lesion development, triglycerides alone are probably not responsible for the lesion development in the L group. The difference between the G group and the L+G hamsters seems to be in the lipoprotein fractions. Therefore there may be a critical level of LDL-C below which triglyceride-rich lipoproteins do not increase the risk of atherosclerosis.

Lipolytic products of triglyceride-rich lipoproteins, such as free fatty acids (FFAs), have also been shown to increase the permeability of endothelial monolayers, enhance lipid accumulation in SMCs, promote endothelial production of inflammatory mediators (NF-*k*B, IL-8, ICAM-1), and alter the extracellular matrix synthesis in cultured SMCs. Studies with both endothelial cells [305] and SMCs [327] have demonstrated that

increased fatty acids also increased oxidative stress and can increase NF-*k*B activation and the expression of NF-*k*B mediated genes including VCAM-1.

Fatty liver, which was evident in both the L and the L + G groups, are linked to insulin resistance. As a result of insulin resistance, there is an excess release of free fatty acids (FFAs) into the circulation leading to more FFA delivery to the liver. The liver converts the increased FFAs to TG which it then incorporates into an increased number of VLDL particles. That free fatty acids could also cause insulin resistance was first proposed by Randle et al., in 1963 [328]. In fact it was more recently determined that insulin resistance is more important than triglyceride levels in causing endothelial dysfunction since hypertriglyceridemia in the absence of severe insulin resistance does not appear to cause endothelial dysfunction [329].

Insulin resistance could also be a factor in the accelerated lesion development of the L+G group. At the 20 week timepoint there was no difference in insulin levels between the G group and the N group or between the L group and the N group. However, the L+G group had significantly elevated insulin over all 3 of these groups. Our findings suggest that the combination of hyperglycemia, insulin resistance, and the increased fatty acids seen in the hyperglycemic/hyperlipidemic F_1B hamster may contribute to endothelial dysfunction and increased atherosclerosis. Moreover, this effect may be considered additive or synergistic.

Plasma Lipid Hydroperoxides. Increased plasma lipid peroxidation products have been observed in both Type I and Type II human diabetics [330, 331]. Previous studies have demonstrated increased levels of plasma peroxides in the combined hyperlipidemic, hyperglycemic hamsters of the APA strain, sometimes almost double the normal

concentration [280, 295, 332]. In this study the L+G group had significantly elevated lipid hydroperoxides over the three other groups. The G group did not have elevated lipid hydroperoxides and this could serve as another difference between the G and L+G groups that may be contributing to the accelerated atherosclerosis seen only in the L+G group.

TG/HDL ratio. The LDL levels of diabetics are often similar to non-diabetics but the LDL that they have are denser and have a higher affinity for proteoglycans ex vivo [175, 176]. Epidemiologic studies have demonstrated that small, dense LDL particles are associated with a greater risk of atherosclerosis [333-336].

In this study there was no significant increase in the absolute non-HDL-C fraction containing VLDL and LDL between the N and G groups, however, differences in the TG/HDL ratio between these two groups indicate a potential difference in small, dense LDL. Small, dense LDL particles more easily infiltrate the subendothelial space where they aggregate and oxidize more readily than larger LDL particles. At 10 weeks the L group had an almost 3-fold increase in this ratio over the N group indicating that hyperlipidemia alone increased the level of small, dense LDL. The G group had almost a 5-fold increase in this ratio over the N group at the 10 week timepoint indicating that hyperglycemia alone also increases the level of small, dense LDL. When combining hyperlipidemia and hyperglycemia, as in the L+G group, this ratio was increased 34.5-fold over the N group at 10 weeks indicating that the combination of hyperlipidemia and hyperglycemia had a synergistic effect in increasing small, dense LDL. At 20 weeks the L group had a 1.7-fold increase, the G group a 3–fold increase and the L+G group a 10-fold increase in the TG/HDL-C ratio over the N group. This confirms that while the G

group had an increase in small, dense LDL particles the higher levels of cholesterol seen in the L+G group further increased the amount of small, dense LDL particles possibly contributing to the accelerated atherosclerosis seen in the L+G group.

MDA and LOX-1 in the aortic arch. In this study the presence of immunoreactivity for MDA and LOX-1 in the aortic arch of the hamsters were utilized as indicators of *in situ* oxidation. The oxidative modification of lipoproteins could be one of the mechanisms for the early development of atherosclerosis in diabetics. Diabetic patients have been generally described as being under enhanced oxidative stress [337] as indicated by their decreased plasma levels of vitamin E and C [338], and their increased serum MDA [172], and urinary F_2-isoprostane [173]. This assertion is also supported by the presence of elevated levels of auto-antibodies to ox-LDL in type II diabetics [174].

The source of free radicals in diabetes is not completely understood. Endothelial cells may increase production of superoxide when exposed to glucose [339-341]. Glycated proteins are also known to lead to free radical formation causing oxidative stress by releasing superoxide [342] and hydrogen peroxide and activating macrophages through RAGE [343].

Many animal studies have shown a correlation of lipid oxidation as measured in blood, urine or vessel wall extracts with the development of atherosclerotic lesions [309]. MDA is a highly reactive dialdehyde produced as a byproduct of PUFA peroxidation and arachindonic acid metabolism. Initially lipid peroxides are formed when PUFAs are attacked by free radicals. The free radicals abstract an electron from the PUFAs during this process. The peroxides react with neighboring PUFAs until they encounter an electron donor such as alpha-tocopherol or other antioxidant. This chain reaction of

PUFAs can compromise cell membrane integrity and lead to cell death. In addition, when reacted with metal ions, such as iron or copper, lipid peroxides may form toxic aldehydes [344] such as MDA and 4-hydroxynonel (4-HNE). MDA has been found in atherosclerotic plaques and may be a critical factor in the endothelial cell dysfunction caused by FFAs [134].

In addition, aldehydes readily combine with functional groups on proteins, lipoproteins and DNA. MDA may modify the apolipoprotein B-100 particle on LDL which is normally recognized by the LDL receptor. In a previous study the LDL in diabetic hamsters was demonstrated to be more susceptible to oxidation than the LDL in non-diabetic hamsters [345]. The apoB-100 particle can also be modified via glycation in the presence of high glucose concentrations [346]. Therefore a person with uncontrolled hyperglycemia already has an increased risk for apoB modification and atherosclerosis [347] which may further be exacerbated by the presence of elevated aldehydes.

In this study MDA was present in the SMCs and ECs of the G group but appears localized to the foam cells of the L group. These two groups have similar triglyceride and insulin levels and small, dense LDL as indicated by the TG/HDL-C ratios. The L group had significantly higher TC, non-HDL-C, HDL-C, and TC/HDL-C ratio and while not significant higher levels of lipid hydroperoxides than the G group. While MDA in the aortic arch was not quantitated there was increased plasma LDL levels in the L group that could potentially be modified by MDA and consequently taken up by LOX-1 on ECs and macrophages in a non-saturation dependent manner.

The acceleration of atherosclerosis in the L+G group could be due to further modification of the apo-B100 particle by glycation which may further induce LOX-1.

The induction of LOX-1 is regulated by many factors involved in atherogenesis. One of the major mechanisms by which LOX-1 upregulation might induce atherogenesis is through its induction of endothelial dysfunction which may involve the activation of NF-*k*B at the transcriptional level [348]. The upregulation of LOX-1 in diabetics may be caused by their increase in fatty acids, particularly linoleic acid which is increased in LDL subfractions in patients with Type II diabetes. Linoleic acid has been demonstrated to increase LOX-1 expression in human aortic endothelial cells [349]. Remnant lipoproteins, which are also increased in diabetics, have been demonstrated to increase monocyte adhesion to human umbilical vein endothelial cells (HUVECS) through LOX-1 receptor-coupled NF-*k*B-dependent nuclear transcription. NF-*k*B, when activated, upregulates proinflammatory gene expression, such as adhesion molecules including VCAM-1, ICAM-1, E-selectin and MCP-1 [350] and LOX-1 [351]. EC expression of C-reactive protein (CRP), an acute inflammatory marker, may also enhance endothelial LOX-1 expression and in doing so promote endothelial dysfunction [204]. TNF-*a*, a mediator of the acute phase response in inflammation, also upregulates LOX-1 expression in SMCs in advanced atherosclerosis in a dose- and time-dependent manner [352]. It has recently been demonstrated that oxLDL may up-regulate NF-*k*B to a certain extent but higher concentrations of oxLDL may actually inhibit NF-*k*B activation [351].

In this study LOX-1 immunoreactivity was observed in foam cells and SMCs in the G group. Increased immunohistochemical staining of MDA in the SMCs was also evident and could therefore be responsible for the up-regulation of LOX-1 in the glycemic hamster. While MDA is also expressed in the L group it is localized more to the foam cells than the SMC. LOX-1 however is expressed not only in the foams cell but

also in the SMCs of the L group. This indicates that LOX-1 might be induced by something other than oxLDL in the SMCs of the L group such as TNF-*a*.

CHAPTER VII

CONCLUSIONS

Hyperglycemia induced LOX-1 and MDA in the Syrian F_1B hamster. Hyperlipidemia also induced the expression of LOX-1 and MDA. The combination of hyperglycemia and hyperlipidemia may have increased the expression of both LOX-1 and MDA. Quantitative analysis of LOX-1 would be important to determining whether LOX-1 may be a potential target in the treatment and prevention of accelerated atherosclerosis in diabetics. The L+G group also have significantly elevated plasma lipid hydroperoxides over the G group and this could also have contributed to their accelerated atherosclerosis. However, a previous hamster study demonstrated that decreasing plasma lipid peroxides and cholesterol oxidation products had no effect on fatty streak lesion formation [345] therefore accelerated atherosclerosis in this model may not be dependent on the extent of LDL oxidation and oxidative stress. It is therefore important to further investigate the effects of LOX-1 on endothelial dysfunction in order to determine whether this may be a mechanism by which LOX-1 might contribute to accelerated atherosclerosis in diabetics.

It appears that the accelerated atherosclerosis seen in the L+G hamsters was mainly associated with their increased TC and non-HDL-C and not the effects of elevated glucose. In fact clinical trials have documented that reduction of LDL-C is associated with a significant reduction in risk for atherosclerosis among diabetic persons while

reduction of blood glucose levels has a relatively small effect on macrovascular atherosclerosis [353].

A recent study concluded that diabetes is a strong predictor of severe but not of mild or moderate progression of atherosclerosis in humans [354]. Our findings suggest that the L+G Syrian F_1B hamster is a useful model for determining the mechanism(s) involved in the development of accelerated atherosclerosis under the conditions of hyperglycemia. The results of this study indicated that while hyperglycemia alone is probably not a cause of atherosclerosis in the Syrian F_1B hamster it may contribute to the development of atherosclerosis when combined with elevated numbers of LDL particles and small dense LDL particles [355]. Kondo et al., recently proposed that there may be a critical size of small dense LDL-C (25.5 nm) above which triglyceride-rich lipoproteins do not increase the risk of atherosclerosis and that there is a critical level of TG (1,500 mg/L) above which LDL is more susceptible to oxidation [356]. While the G group in this study had an increase in small, dense LDL particles, the higher levels of cholesterol and triglycerides seen in the L+G group further increased the amount of small, dense LDL particles possibly contributing to the accelerated atherosclerosis seen in the L+G group. In addition, the TC/HDL-C ratio appeared to be an accurate indicator of the development of atherosclerosis in the Syrian F_1B hamsters since it was significantly elevated in the L+G group but not in the G group.

The elevated insulin levels in the L+G group may also have contributed to their accelerated atherosclerosis. Elevated insulin levels seen in the pre-diabetic phase of Type II diabetes may also be a contributing factor to accelerated atherosclerosis in these patients. Therefore treatment of type II diabetes may be best aimed at not only lowering

LDL-C but elevating HDL, decreasing triglycerides and preventing the hyperinsulinemia that occurs in the pre-diabetic phase when atherogenesis occurs as opposed to treatment aimed at reducing the resulting hyperglycemia [357-359] in the diabetic phase only.

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APPENDICES

APPENDIX A

TABLES

Table 1
Hamster Body Weights

Group/Weeks	Initial Weight	Final Weight
N/10	96.3 ± 3.5	100.8 ± 4.6
N/20	94.6 ± 5.6	109.0 ± 7.0*
L/10	94.7 ± 4.5	109.7 ± 6.5**
L/20	97.8 ± 4.9	108.0 ± 6.5
G/10	96.2 ± 3.7	101.3 ± 5.7
G/20	95.2 ± 5.0	104.0 ± 7.7
L+G/10	95.2 ± 4.1	101.9 ± 6.4
L+G/20	96.5 ± 4.4	107.1 ± 7.9

Mean (± SD) body weight (g). *Significant increase ($p<0.01$) over initial weight. **Significant increase ($p<0.001$) over initial weight and significant increase ($p<0.05$) over N/10 final weight.

Table 2

Hamster Lipid, Glucose, and Insulin Values

Group/ Weeks	TC (mg/dL)	HDL-C (mg/dL)	Non-HDL-C (mg/dL)	TG (mg/dL)	TC/HDL-C	Ave. TG/ Ave. HDL	Glucose (mg/dL)	Insulin (ng/ml)
N / 10	72.94 ± 7.42	47.52 ± 4.95	25.48 ± 6.86	52.40 ± 14.5	1.540 ± 0.2	1.1	178.4 ± 26.2	0.039 ± 0.251
N / 20	90.97 ± 14.1	48.32 ± 7.54	42.65 ± 12.0	109.2 ± 32.2	1.917 ± 0.3	2.3	205.7 ± 50.5	0.134 ± 0.114
L / 10	318.6 ± 51.9	71.25 ± 8.90	247.4 ± 49.5	226.0 ± 107	4.486 ± 0.8	3.2	285.2 ± 35.2	0.160 ± 0.095
L / 20	386.4 ± 82.1	75.21 ± 14.4	311.2 ± 85.6	301.7 ± 131	5.329 ± 1.8	4	244.1 ± 47.4	0.136 ± 0.051
G / 10	85.76 ± 19.3	46.49 ± 5.99	39.27 ± 13.7	244.5 ± 89.7	1.829 ± 0.2	5.3	385.8 ± 33.9	0.224 ± 0.076
G / 20	73.52 ± 16.5	34.22 ± 1.24	39.30 ± 16.7	232.7 ± 100	2.150 ± 0.5	6.8	360.7 ± 32.9	0.256 ± 0.079
L+G / 10	2170 ± 321	207.7 ± 119	2020 ± 267	7901 ± 1756	12.28 ± 3.5	38	392.7± 84.2	7.154 ± 3.381
L+G / 20	2491 ± 941	309.4 ± 57.5	2320 ± 997	7271 ± 4159	8.425 ± 3.1	23.2	434.8 ± 124	1.043 ± 0.069

Mean ± SD assay values

Table 3
Significant Differences in Lipids, Glucose, and Insulin at Week 10

Groups Compared	TC (mg/dL)	HDL-C (mg/dL)	Non-HDL-C (mg/dL)	TG (mg/dL)	TC/ HDL-C	Glucose (mg/dL)	Insulin (ng/ml)
N vs L	***	*	***	***	***	**	*
N vs G	NS	NS	NS	***	NS	***	***
N vs L+G	***	***	***	***	***	***	***
G vs L	***	*	***	NS	***	$\underline{**}$	NS
L vs L+G	***	***	***	***	***	**	***
G vs L+G	***	***	***	***	***	NS	***

*P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant. $\underline{**}$ indicates significant decrease of the second group. All others indicate significant increase of the second group.

Table 4
Significant Differences in Lipids, Glucose, and Insulin at Week 20

Groups Compared	TC (mg/dL)	HDL-C (mg/dL)	Non-HDL-C (mg/dL)	TG (mg/dL)	TC/ HDL-C	Glucose (mg/dL)	Insulin (ng/ml)
N vs L	***	***	***	*	**	NS	NS
N vs G	NS	**	NS	*	NS	***	NS
N vs L+G	***	***	***	***	***	***	***
G vs L	***	***	***	NS	*	$\underline{*}$	*
L vs L+G	***	***	***	***	*	***	***
G vs L+G	***	***	***	***	***	NS	***

P < 0.05, ** P < 0.01, *** P < 0.001, NS = not significant. $\underline{*}$ indicates Significant decrease of the second group. All others indicate significant increase of the second group.

APPENDIX B

FIGURES

Figure 1
Experimental Design

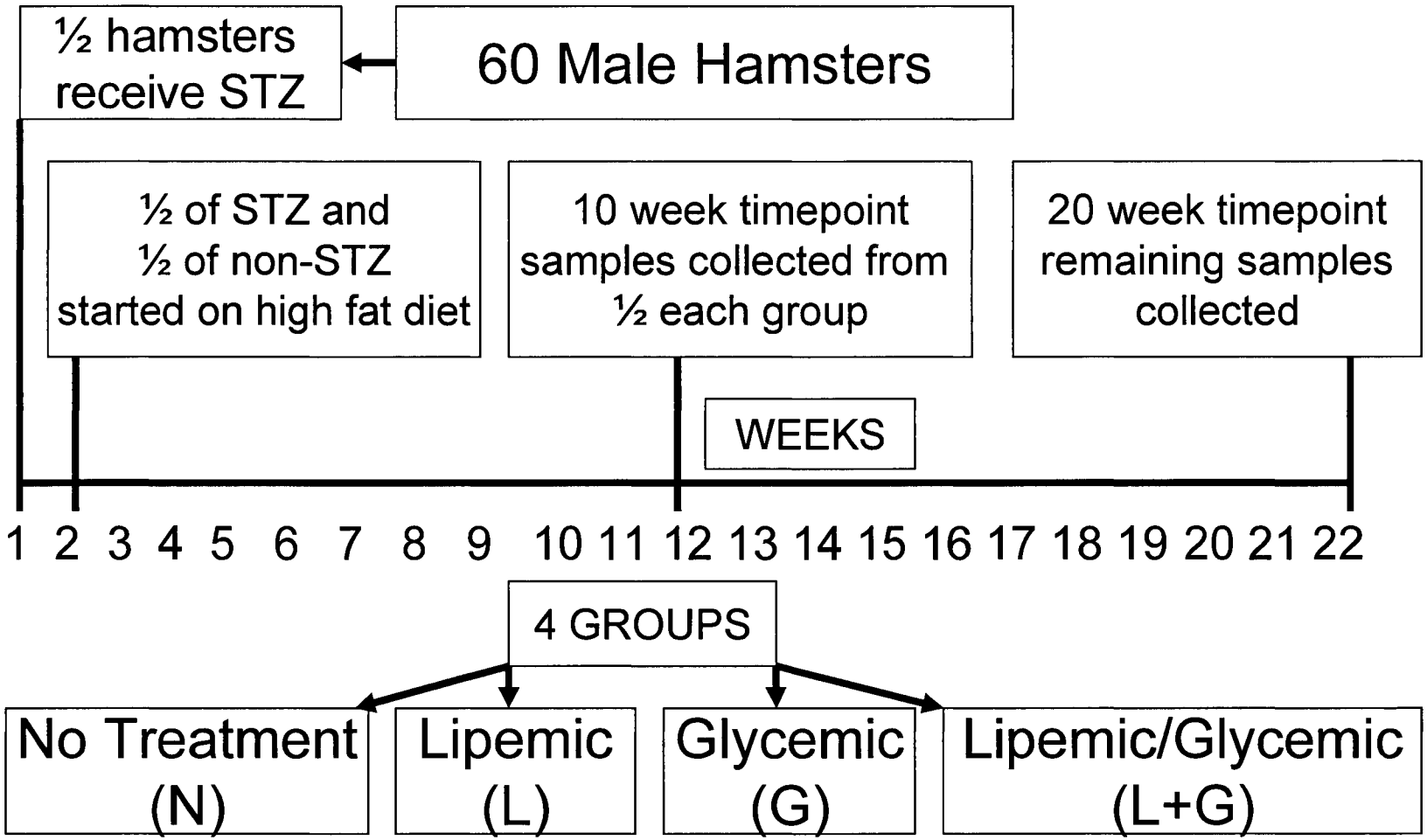


Figure 2
Normal Hamster Pancreatic Insulin Immuno-reactivity

Sections of normal hamster pancreas at 10 weeks. Purple chromogen deposition indicates immuno-reactivity with insulin. 200X.

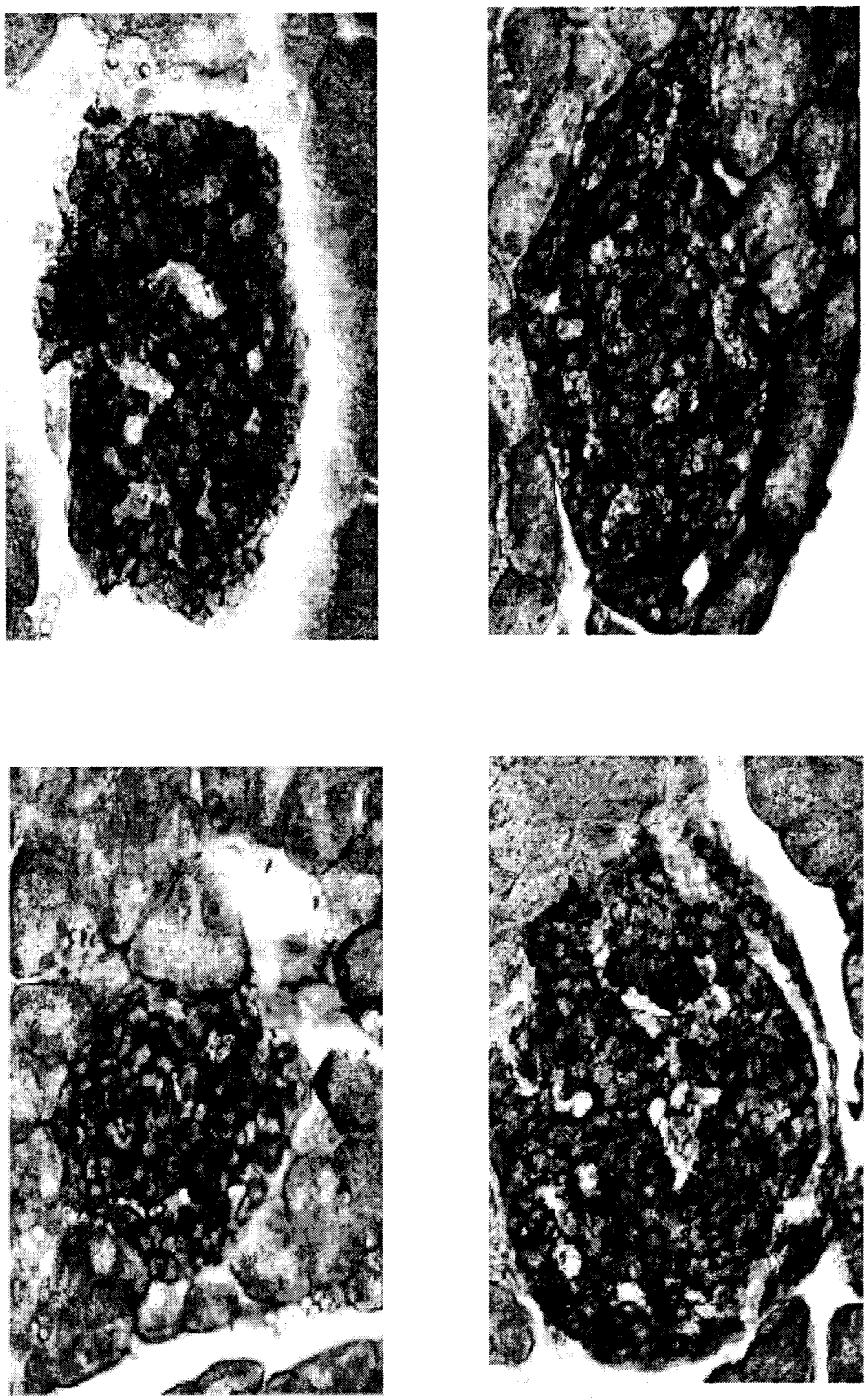


Figure 2 – Normal Hamster Pancreatic Insulin Immuno-reactivity

Figure 3
Glycemic Hamster Pancreatic Insulin Immuno-reactivity

Sections of glycemic hamster pancreas at 10 weeks. Purple chromogen deposition indicates immuno-reactivity with insulin. 200X.

Figure 3 – Glycemic Hamster Pancreatic Insulin Immuno-reactivity

Figure 4
Hamster Aortic Arch Lesion Development

Cross sections of hamster aortic arch at 20 weeks. N represents non-lipidemic/non-glycemic hamsters, G represents glycemic hamsters, L represents lipidemic hamsters and L + G represents lipidemic/glycemic hamsters. 200X.

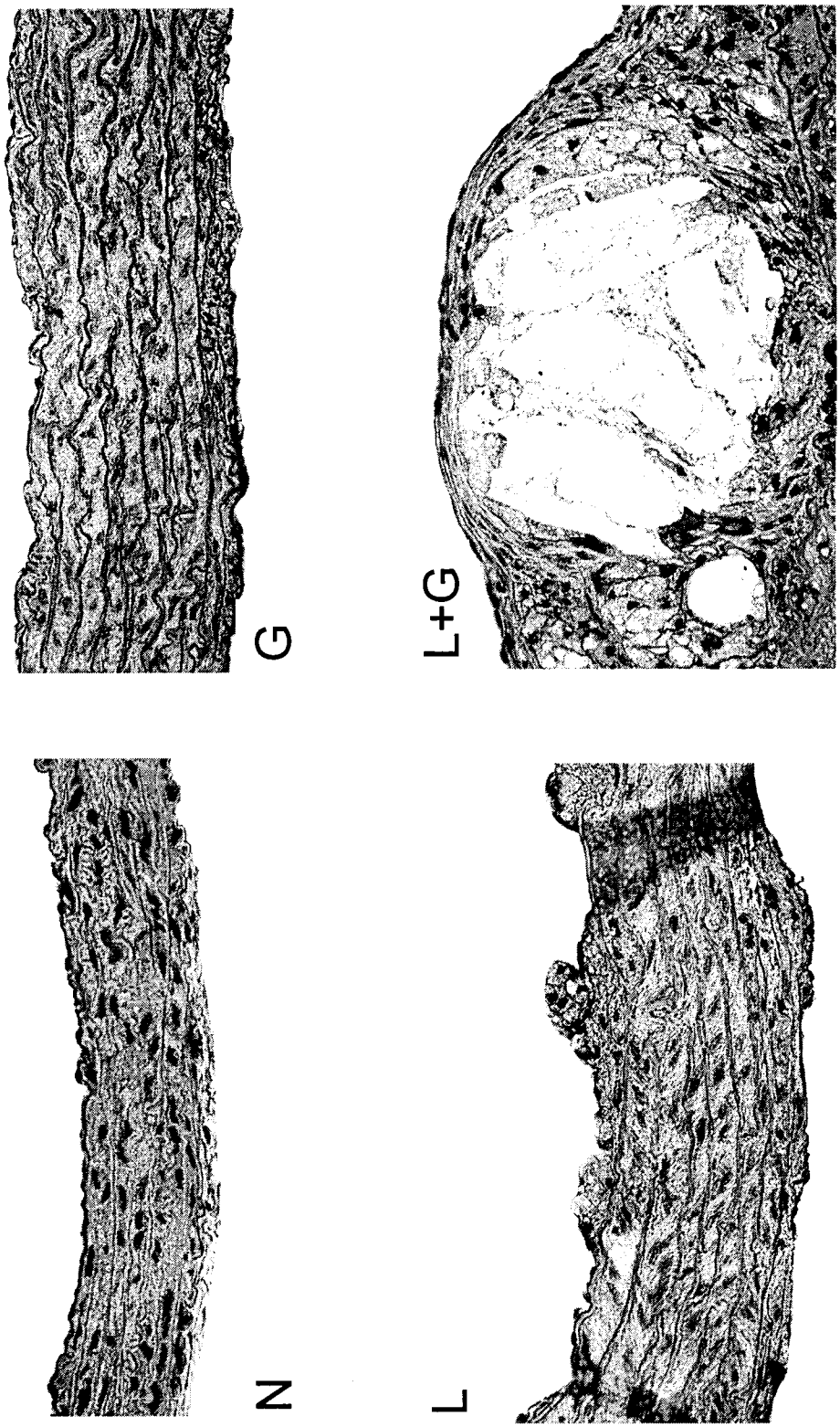


Figure 4 – Hamster Aortic Arch Lesion Development

Figure 5
Hamster Aortic Arch MDA Immuno-reactivity

Cross sections of hamster aortic arch at 20 weeks. N represents non-lipidemic/non-glycemic hamsters, G represents glycemic hamsters, L represents lipidemic hamsters and L + G represents lipidemic/glycemic hamsters. Red chromogen deposition indicates immuno-reactivity with MDA. 200X.

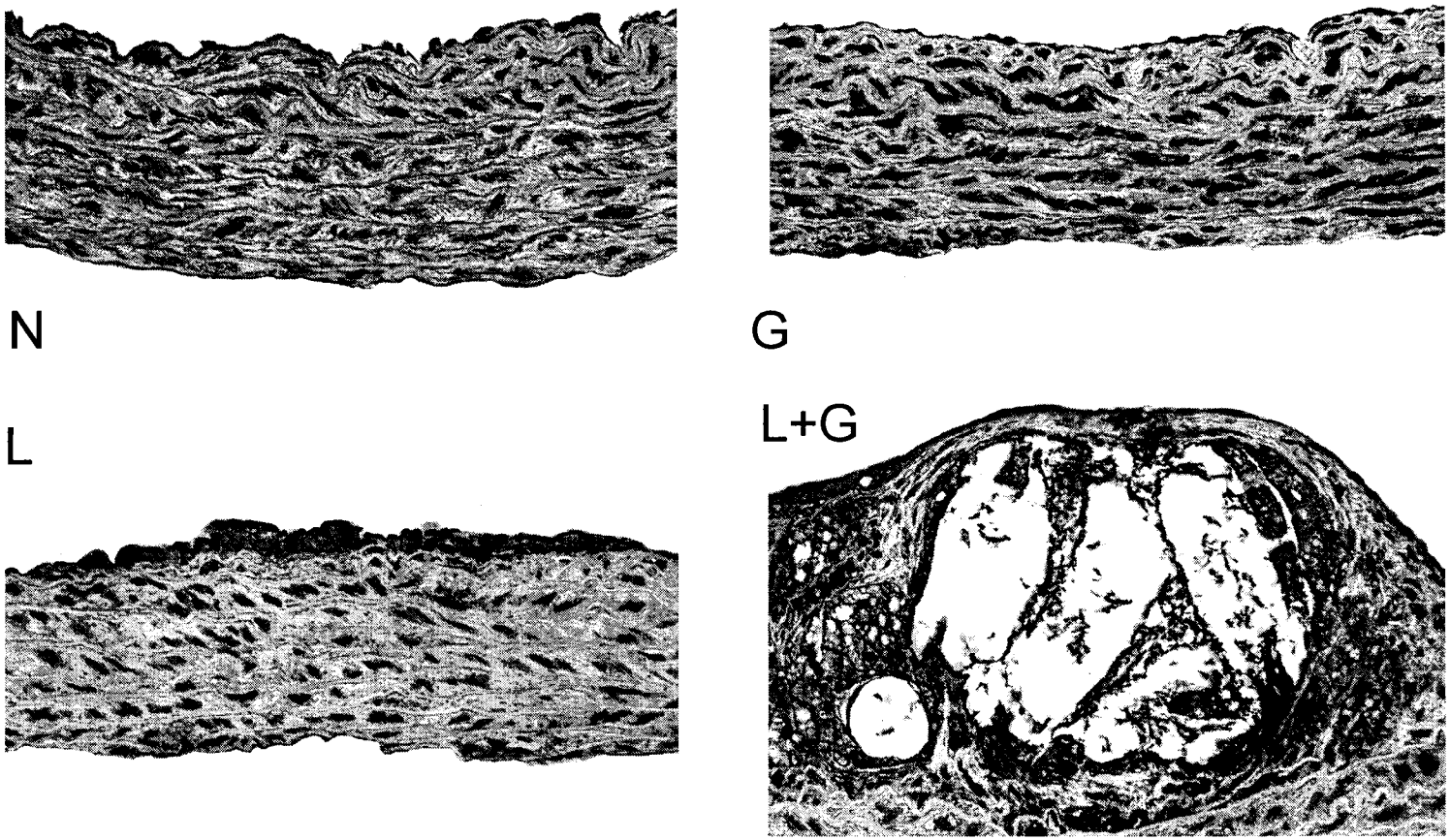


Figure 5 - Hamster Aortic Arch MDA Immuno-reactivity

Figure 6
Hamster Aortic Arch LOX-1 Immuno-reactivity

Cross sections of hamster aortic arch at 20 weeks. N represents non-lipidemic/non-glycemic hamsters, G represents glycemic hamsters, L represents lipidemic hamsters and L + G represents lipidemic/glycemic hamsters. Red chromogen deposition indicates immuno-reactivity with LOX-1. 200X.

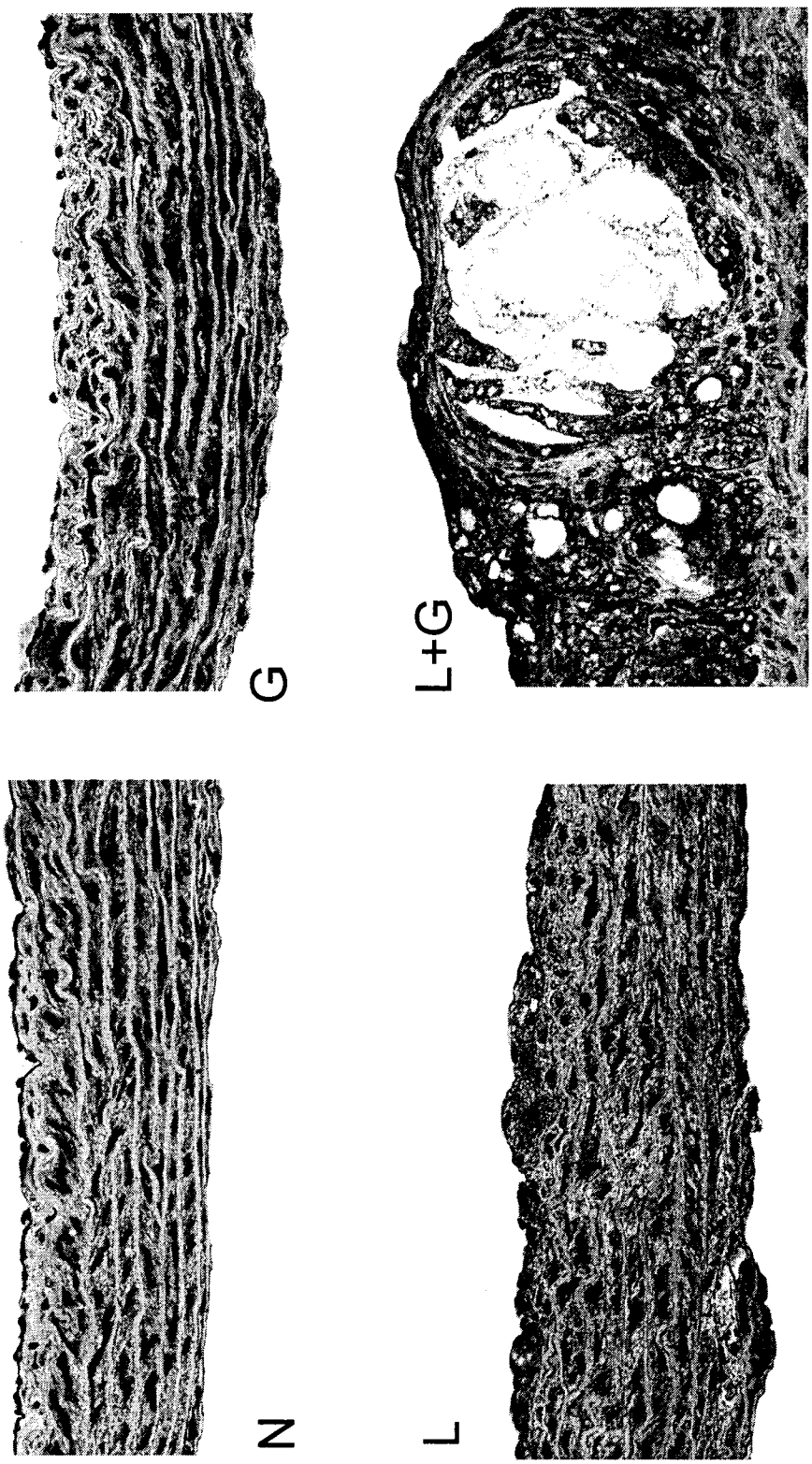
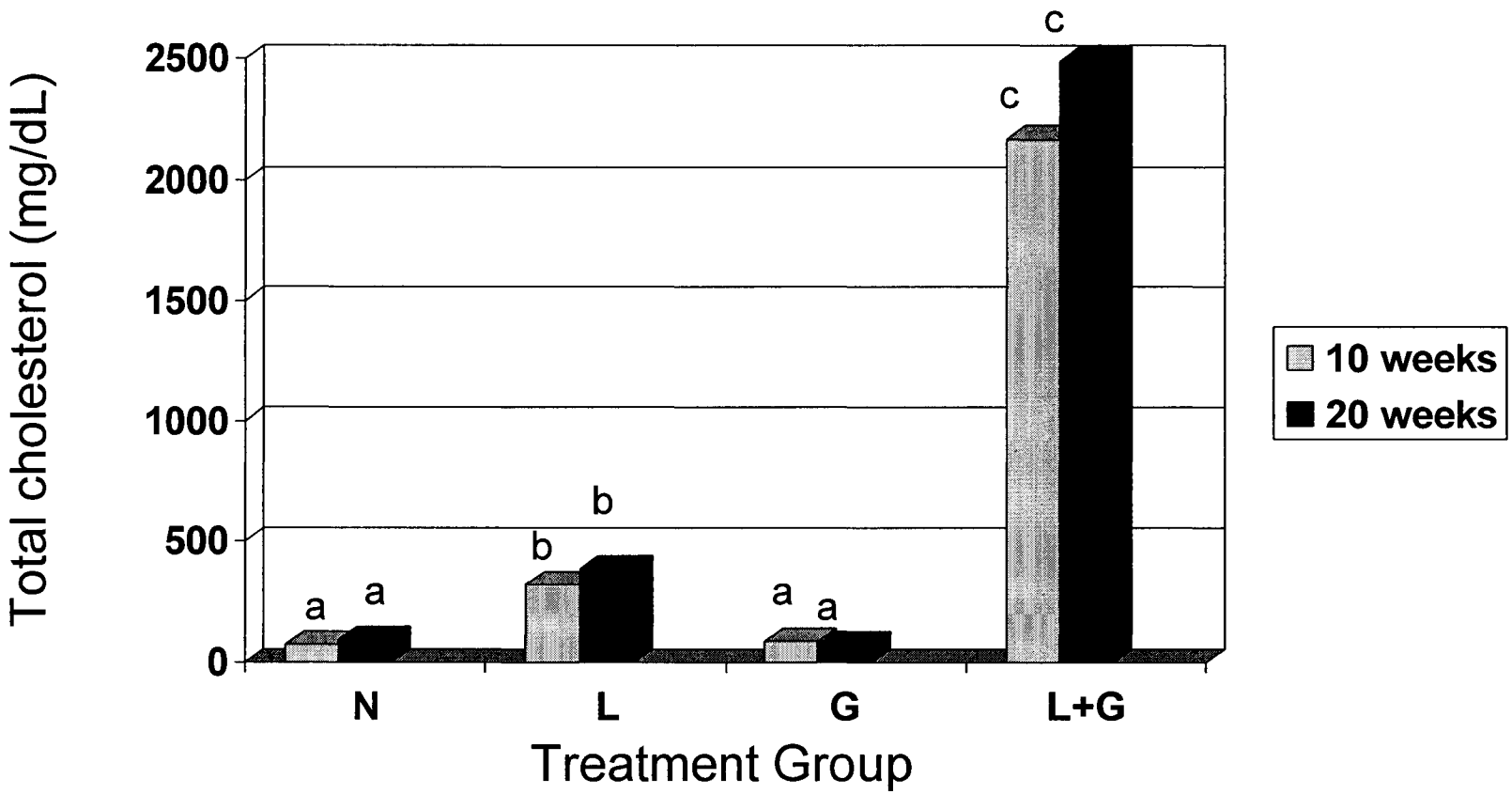


Figure 6 – Hamster Aortic Arch LOX-1 Immuno-reactivity

APPENDIX C

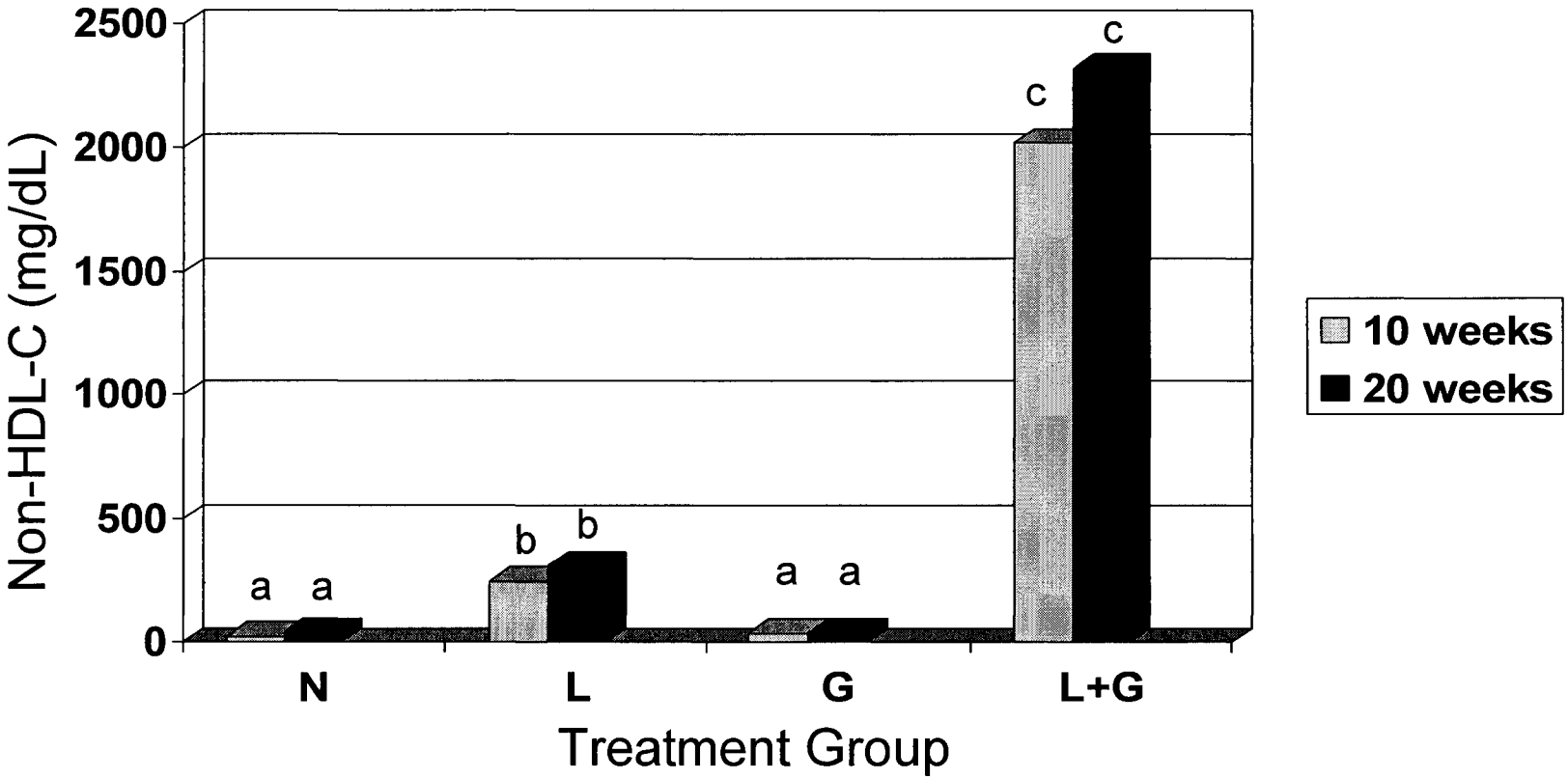
GRAPHS

Graph 1
Total Cholesterol



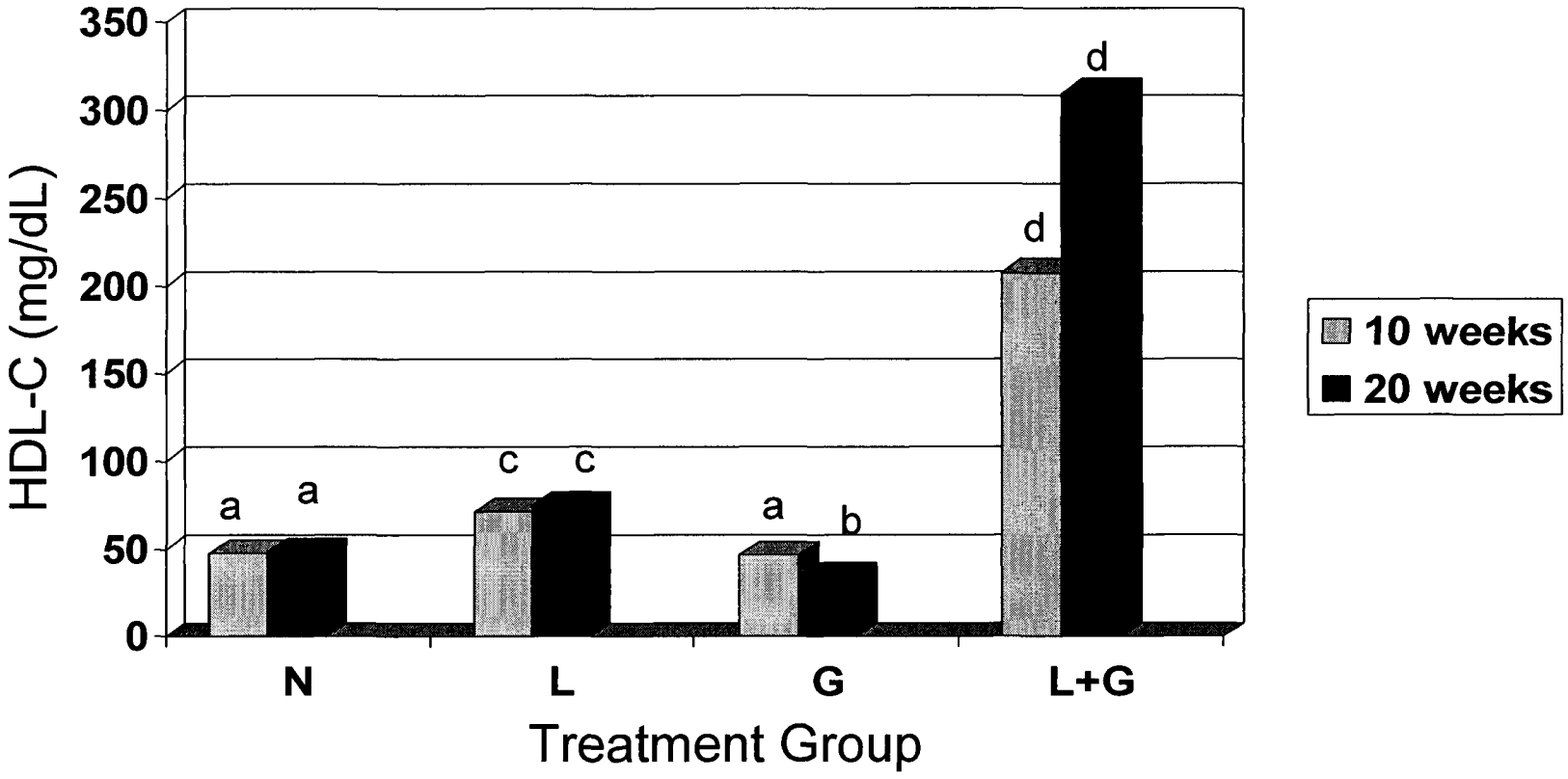
b significantly increased over a. c significantly increased over b and a.

Graph 2
Non-HDL-C



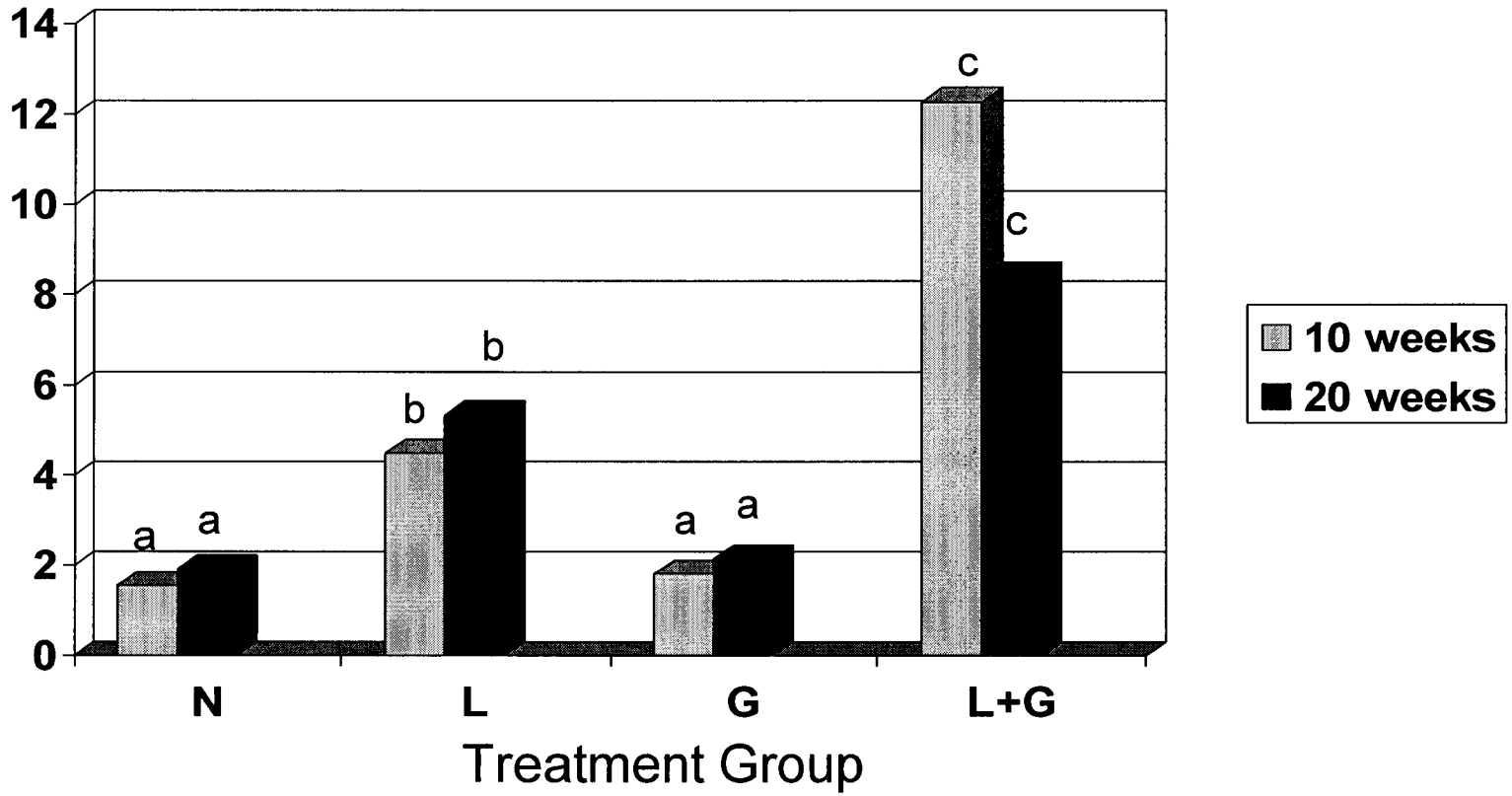
b significantly increased over a. c significantly increased over a and b.

Graph 3
HDL-C



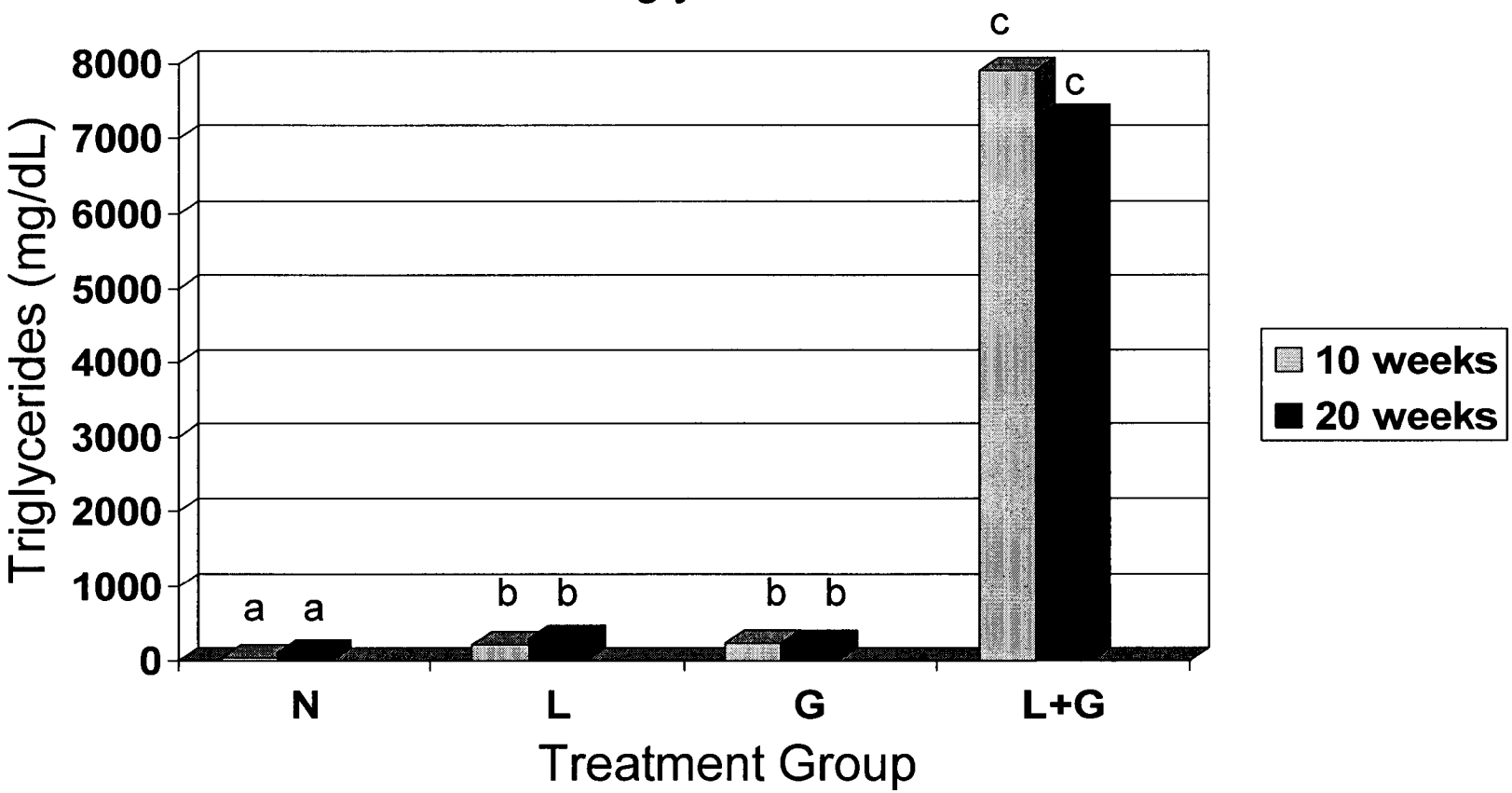
c significantly increased over a. b significantly decreased below a. d significantly increased over a, b and c.

Graph 4
TC/HDL-C

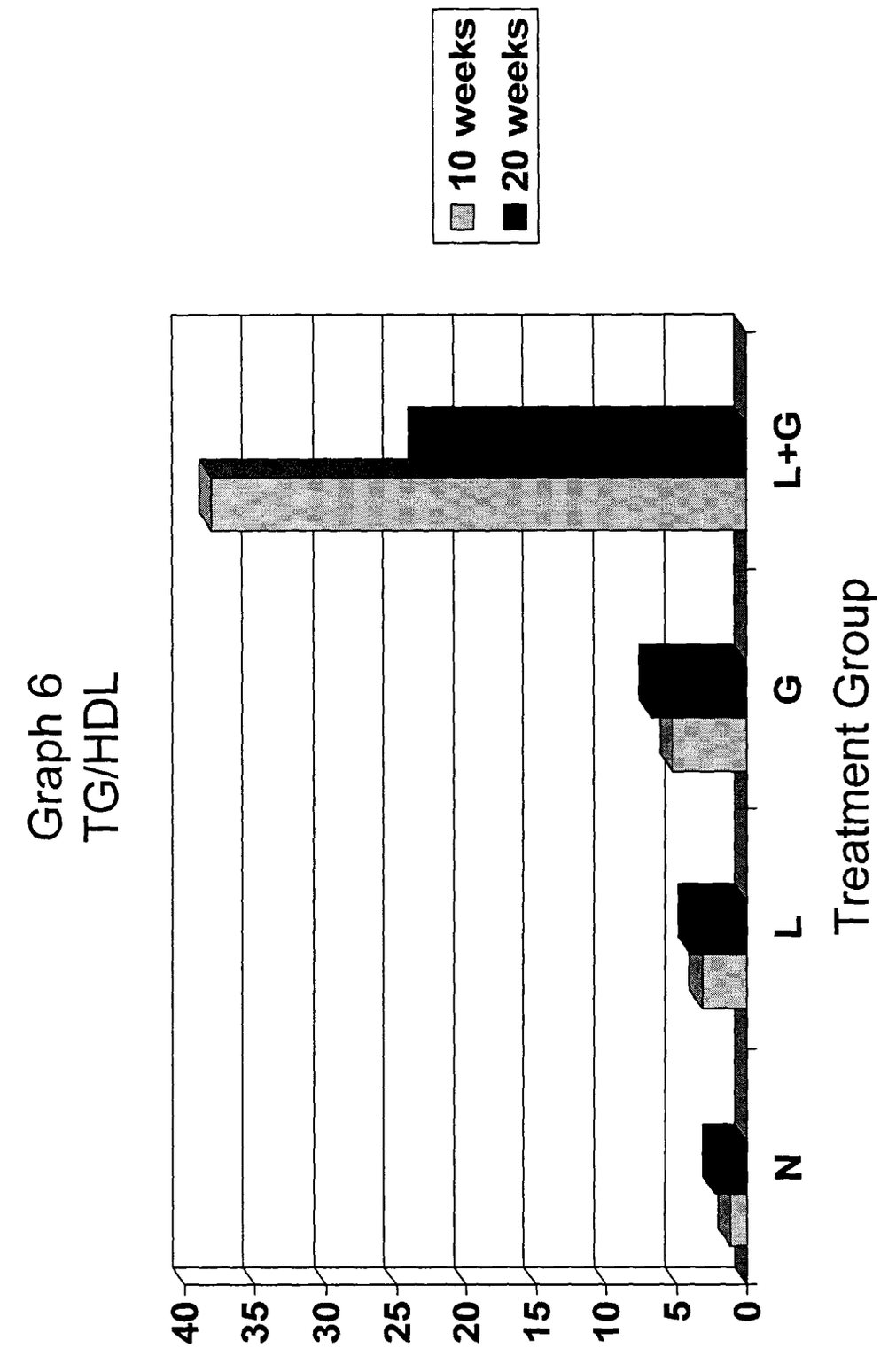


b significantly increased over a. c significantly increased over a and b.

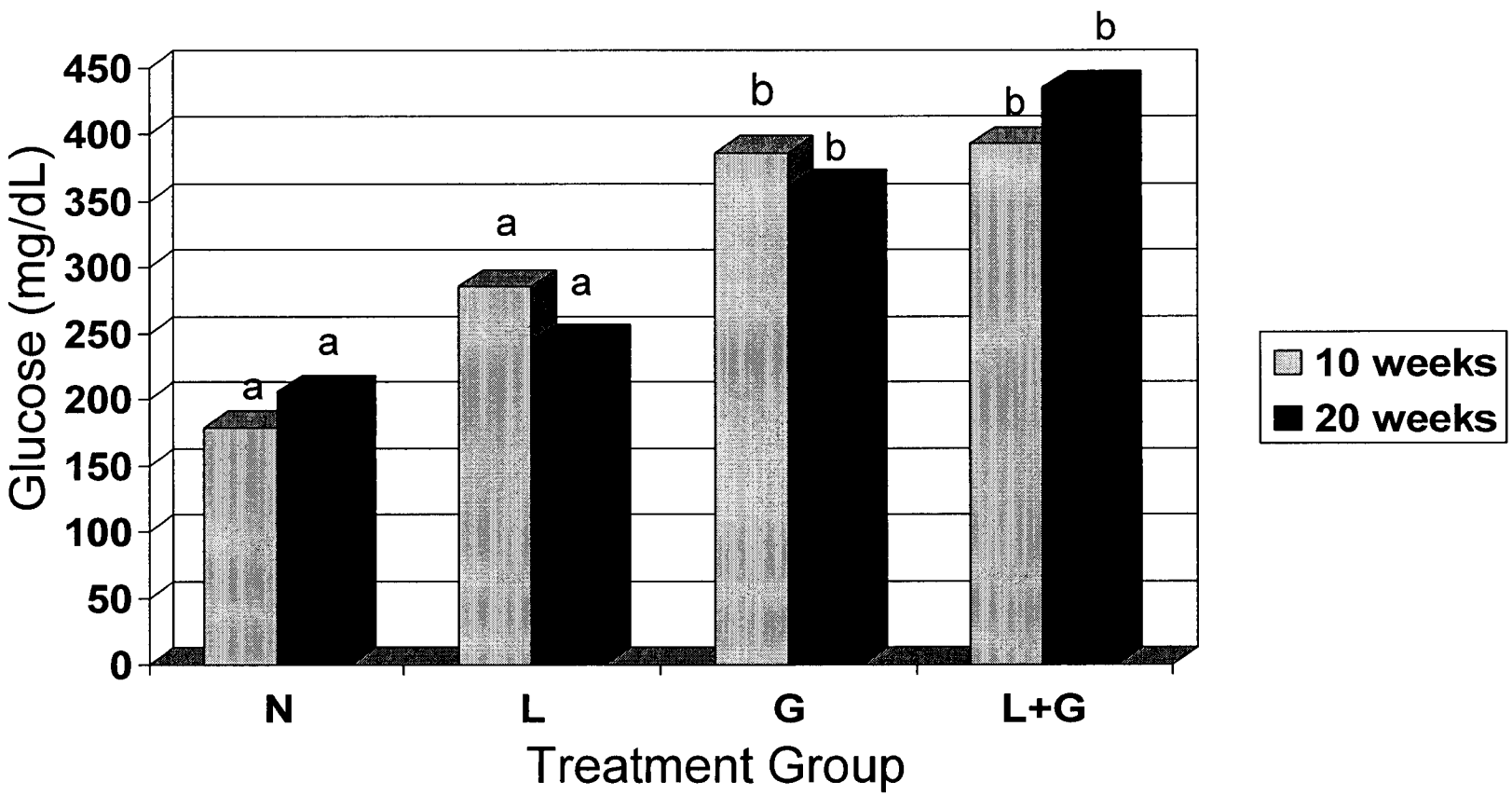
Graph 5
Triglycerides



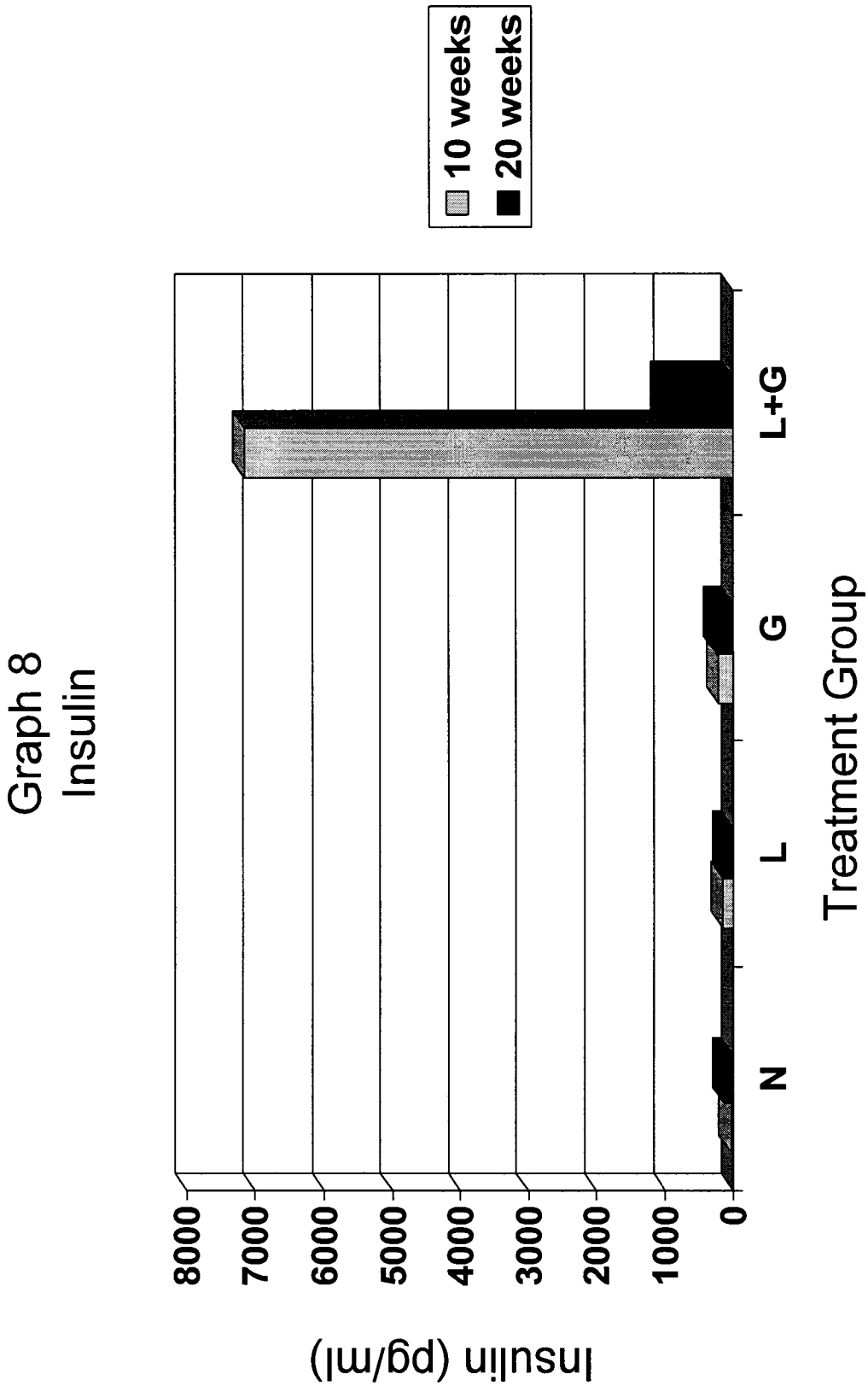
b significantly increased over a. c significantly increased over b.

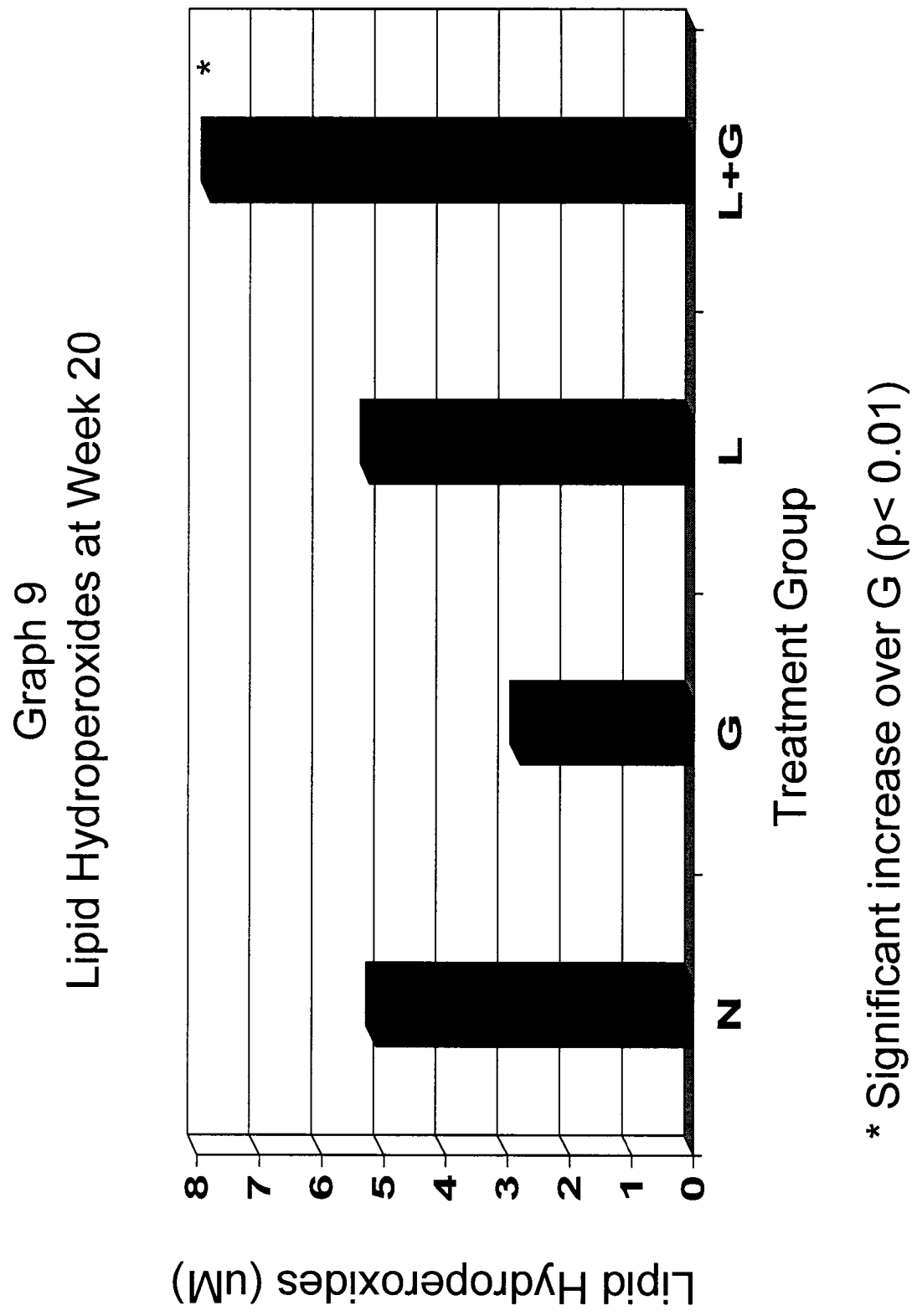


Graph 7
Glucose

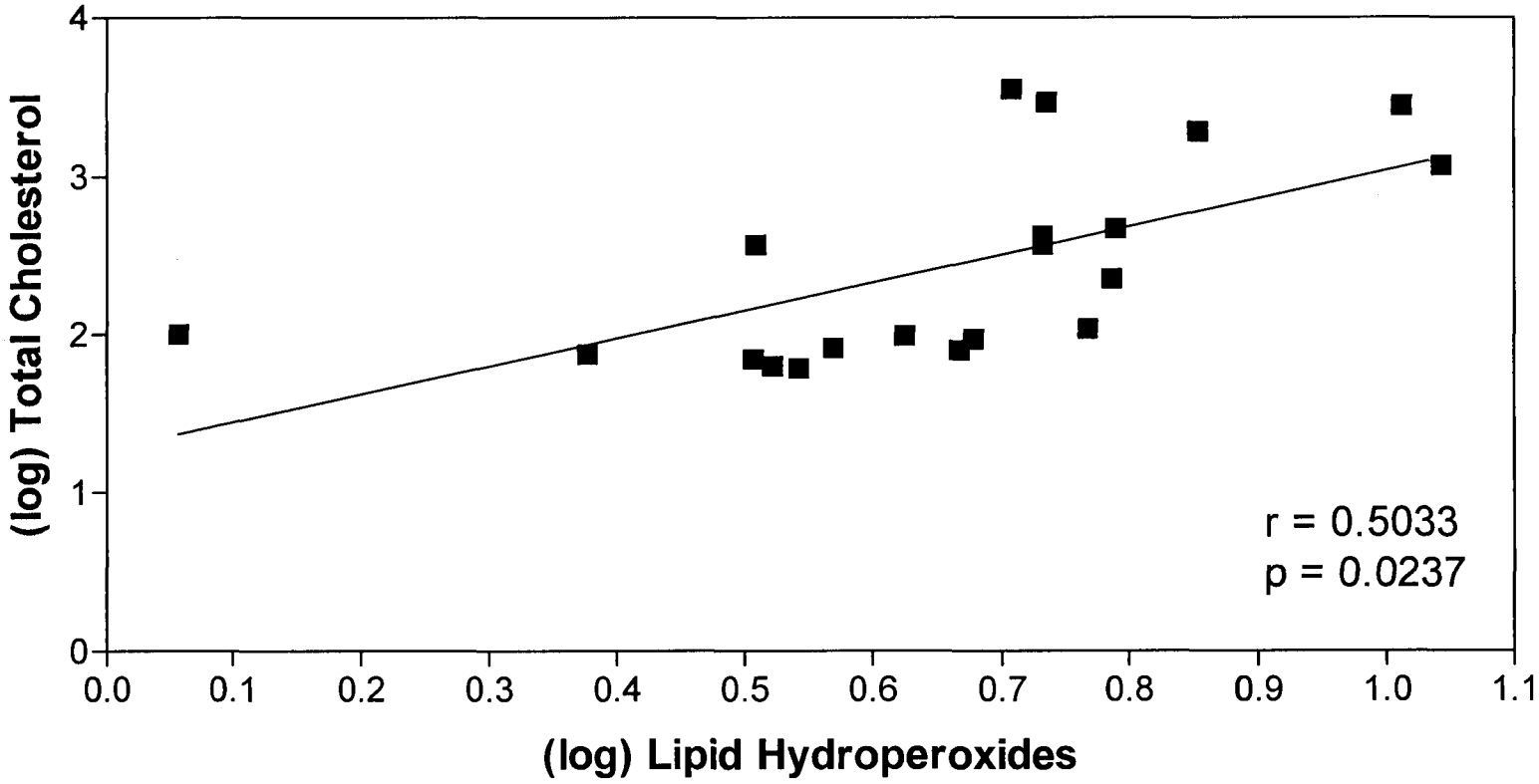


b significantly increased over a.





Graph 10
Correlation between total cholesterol and lipid hydroperoxides in all hamsters at week 20.



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LAST NAME	Foxall	**FIRST NAME**	Thomas
DEPT	Animal and Nutritional Sciences, Kendall Hall	**APP'L DATE**	8/7/2001
OFF-CAMPUS ADDRESS (if applicable)	Animal and Nutritional Sciences, Kendall Hall	**IACUC #**	010702
		REVIEW LEVEL	3
		TODAY'S DATE	8/8/2001
PROJECT TITLE	Role of Oxidized LDL Receptors (LOX-1) in Atherogenesis in Normal and Diabetic Animals		

All cage, pen or other animal identification records must include your IACUC Protocol # as listed above.

The Institutional Animal Care and Use Committee has reviewed and approved the protocol submitted for this study under Category 3 on Page 3 of the "Application for Review of Animal Use or Instruction Protocol" - the research involves chronic maintenance of animals with a disease/functional deficit and/or procedures potentially inducing moderate pain, discomfort or distress which will be treated with appropriate anesthetics/analgesics.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please note: Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. *Participation is mandatory* for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Van Gould at 862-4629 or Julie Simpson at 862-2003.

For the Institutional Animal Care and Use Committee,

Roger E. Wells, D.V.M.
Acting Chair

cc: File